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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

MICRORNAS PROFILE: A PROMISING ANCILLARY TOOL FOR ACCURATE RENAL CELL TUMOR DIAGNOSIS

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Relevant abbreviations

USA	United States of America
RCT	Renal cell tumor
RCC	Renal cell carcinoma
IARC	international Agency for Research on Cancer
BMI	Body man index
ESRD	end-stage renal disease
ARCD	Acquired renal cystic disease
TCE	Trichloroethylene
VHL	Von Hippel-lindau
FH	Fumarate hydratase
HLRCC	hereditary leiomyomatosis renal cell carcinoma
BHD	Birt-Hogg-Dub
US	Ultrasound
CT	Computed tomography
WHO	World Health organization
AJCC	American Joint Committee on Cancer
IUAC	International Union Against Cancer
TNM	Tumor-node-metastases
TSG	Tumor suppressor genes
ccRCC	Clear renal cell carcinoma
pRCC	Papillary renal cell carcinoma
chRCC	Chromophobe renal cell carcinoma
mRCC	metastatic renal cell carcinoma
FDA	Food and Drug Administration
DNA	Deoxyribonucleic Acid
DNMTs	DNA methyltransferases
SAM	S-adenosyl-L-methionine
MBDPs	methyl-CpG-binding domain proteins
CpG	Cytosine-Phosphate-Guanine
HATs	histone acetyltransferases
HDACs	histone deacetylase
HMTs	histone methyltransferases
HDMs	histone demethylases
ncRNAs	Non-coding RNAs
miRNAs	microRNAs

RNA	Ribonucleic Acid
dsRNA	double-stranded RNA
DGCR8	Di George syndrome critical region 8
SNPs	single nucleotide polymorphisms
XPO5	exportin-5
TARBP2	Trans-activator RNA-binding protein 2
ORF	open reading frame sequence

Summary

Background: Renal cell tumors (RCT) are clinically, morphologically and genetically heterogeneous. Accurate identification of renal cell carcinomas (RCC) and its discrimination from normal tissue and benign tumors is mandatory. We, thus, aimed to define a panel of microRNAs that might aid in the diagnostic workup of RCTs.

Material and methods: Fresh-frozen tissues from 120 RCTs (clear cell RCC, papillary RCC, chromophobe RCC and oncocytomas: 30 cases each), 10 normal renal tissues and 60 cases of *ex vivo* fine-needle aspiration biopsies from RCTs (15 of each subtype – validation set) were collected. Expression levels of miR-21, miR-141, miR-155, miR-183 and miR-200b were assessed by quantitative reverse transcription-PCR (qRT-PCR). Receiver Operator Characteristics (ROC) curves were constructed and the areas under the curve (AUC) were calculated to assess diagnostic performance. Disease-specific survival curves and a Cox regression model comprising all significant variables were computed.

Results: RCTs displayed significantly lower expression levels of miR-21, miR-141, and miR-200b compared to normal tissues and expression levels of all miRs differed significantly between malignant and benign RCTs. Expression analysis of miR-141/miR-200b accurately distinguished RCTs from normal renal tissues, oncocytoma from RCC and chromophobe RCC from oncocytoma. The diagnostic performance was confirmed in the validation set. Interestingly, miR-21, miR-141 and miR-155 expression levels showed prognostic significance, in univariate analysis.

Conclusions: The miR-141/miR200b panel accurately distinguishes RCC from normal kidney and oncocytoma in tissue samples, discriminating from normal kidney and oncocytoma, whereas miR-21, miR-141 and miR-155 convey prognostic information. This approach is feasible in fine-needle aspiration biopsies and might provide an ancillary tool for routine diagnosis.

Resumo

Introdução: Os tumores de células renais (TCR) são clinicamente, morfologicamente e geneticamente heterogéneos. A correcta identificação dos carcinomas de células renais (CCR), bem como a sua discriminação do tecido normal e dos tumores benignos é de extraordinária relevância. Assim, o nosso objectivo foi definir um painel de microRNAs que pudessem auxiliar no diagnóstico de TCRs.

Material e métodos: Cento e vinte tecidos congelados a fresco de casos clínicos randomizados (CCR do tipo células claras, CCR do tipo papilar, CCR do tipo cromófobo e oncocitomas: 30 casos cada), 10 tecidos renais normais foram seleccionados para análise mlecular. Adicionalmente, como série de validação foram analisados 60 casos de biópsias aspirativas por agulha fina *ex vivo* (15 de cada subtipo).. Os níveis de expressão do miR-21, miR-141, miR-155, miR-183 e miR-200b foram avaliados por PCR quantitativo em tempo real (qRT-PCR), após a síntese do cDNA. As curvas ROC *(Receiver Operator Characteristics*) foram construídas e as áreas sob a curva (AUC) foram calculadas para avaliar o desempenho diagnóstico destes miRs. As curvas de sobrevida específica de doença foram calculadas para cada marcador molecular, tendo sido igualmente usado o modelo de regressão de Cox.

Resultados: Os TCRs apresentaram níveis de expressão significativamente mais baixos nos miR-21, miR-141 e miR-200b, em comparação com tecidos normais e os níveis de expressão de todos os miRs diferiram significativamente entre TCRs malignos e benignos. A análise da expressão de miR-141/miR-200b distinguiu com elevadade acuidade os TCRs dos tecidos renais normais e dos oncocitomas, bem como os CCR do tipo cromófobo dos oncocitomas. O desempenho da acuidade diagnóstica deste painel foi confirmado no grupo de amostras de validação. Na análise univariada,. os níveis de expressão dos miR-21, miR-141 e miR-155 demonstraram ter valor de prognóstico.

Conclusões: O painel miR-141/miR200b foi capaz de correctamente distinguir CCR de tecido renal normal e de oncocitoma, discriminando igualmente rim normal de oncocitomas. No entanto os miR-21, miR-141 e miR-155 demonstraram ser informativos quanto ao prognóstico. Esta abordagem é viável em biópsias aspirativas por agulha fina e pode constituir um método auxiliar para o diagnóstico de rotina hospitalar.

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Introduction

1. Kidney cancer

1.1. Epidemiology

Currently, cancer remains the second leading cause of death in both genders in all ages in developed countries. Globally, kidney cancer accounts for approximately 4% of all cancers. In 2008, 273,500 new cases of kidney cancer were diagnosed and 116,300 persons die from this disease worldwide [1].

Currently, in the United States of America (USA), Kidney cancer is the 6th most frequent neoplasm in men and the 8th most frequent in women (Figure 1). Moreover, it is estimate for both sexes that 65,150 new cases of kidney cancer will be diagnosed as well as 13,680 deaths will occur de to this neoplasm [2].

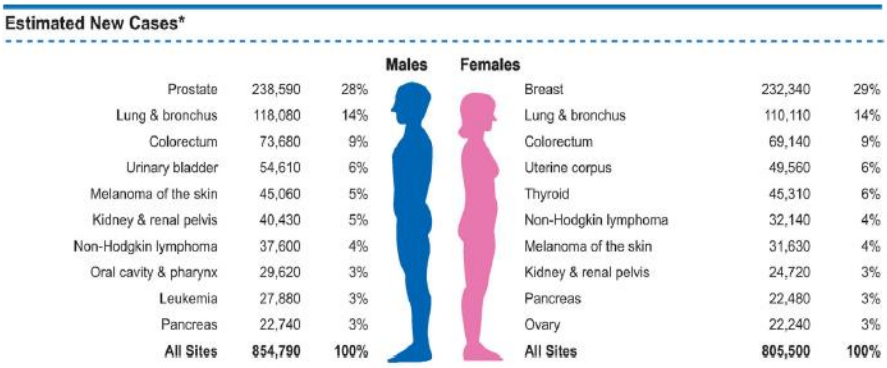

Figure 1: Ten Leading Cancer Types for the Estimated New Cancer Cases by Sex, United States, 2013. *Adapted from* [2].

In Europe, kidney cancer is 7th most common cancer in both sexes and the estimated number of kidney cancer cases and deaths in 2008 was 101,937and 45,096 respectively, (Figure 2) for both sexes. In Portugal, the estimated number of new Kidney cancer cases in 2008 was 443 for males and 248 for females. In the same period, 347 males and females died due to kidney cancer [3].

The incidence of kidney cancer is quite variable across the countries. In 2008, the highest incidence rates were registered in Europe, North America and Australia, whereas the lowest incidence occurs in Asia, South American and Africa (Figure 2) [3]. This incidence rate variation may be attributable to differences in the access to health care, diagnostic imaging, genetic background, and prevalence of lifestyle or environmental risk factors [4].

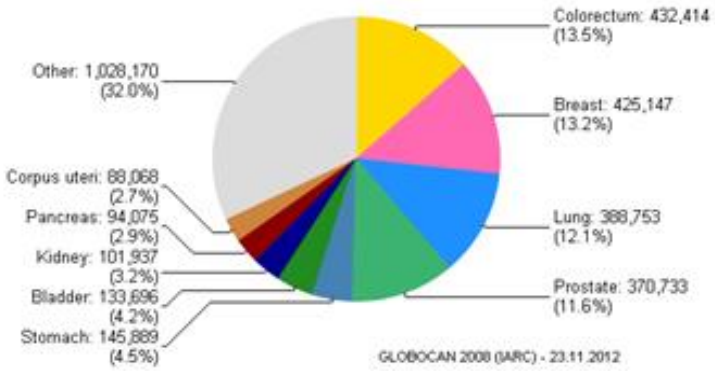

Figure 2: Incidence of different types of cancer in Europe for 2008. Kidney cancer (represented in dark blue) is the 7th most common diagnosed cancer in Europe. *Adapted from* [3].

Renal cell tumors (RCT) represent 90-95% of neoplasms arising from kidney [5]. It is the third most common cancer of the genitourinary tract and the most lethal urological cancer, since more than 40% of patients with renal cell carcinoma (RCC) die of the disease, opposite to the 20% observed for prostate or bladder cancers [6,7].

Incidence rates for RCTs have been steadily increasing in Europe and in the USA for the last 3 decades. Compared to 1971, there has been a 5-fold increase in the incidence and a two-folds increase in mortality from RCTs. Although, the increased usage of imaging has resulted in more RCTs found by incidental detection, it does not entirely explain this augment in incidence [5,6].

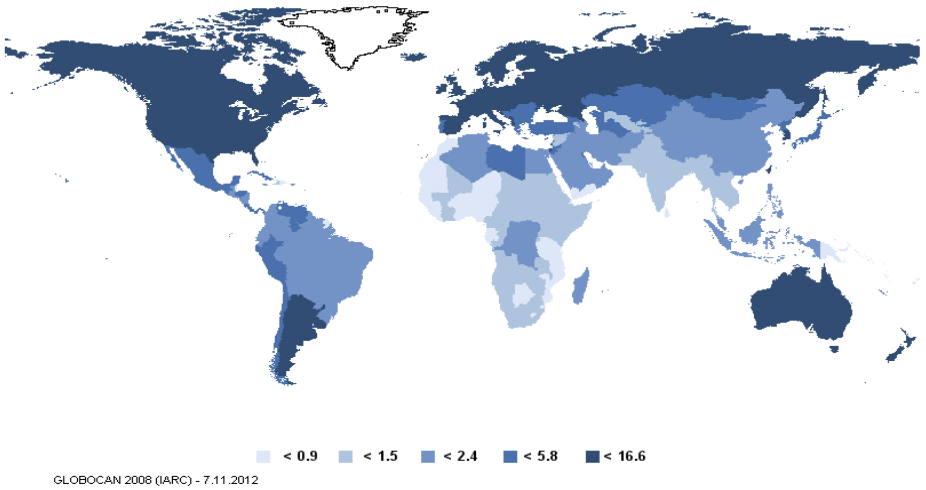

Figure 3: Estimated age-standardized Kidney cancer incidence rate per 100,000, across the world. *Adapted from* [3].

1.2. Risk Factors

Most of the cases (96%) of RCTs are sporadic and only 4% are hereditary [8]. There are multiple factors related to the development of RCTs. Some of them have been demonstrated in experimental models and *in vitro* studies, however only a few have been clearly established as etiologic factors [7]. Indeed, only smoking, obesity and hypertension are well-established factors associated with RCT[9]. Additionally. there are environmental risk factors such as occupational exposure to different chemicals, radiation and renal dialysis, that have been linked to RCT, nonetheless without a solid scientific/epidemiological support[10].

1.2.1. Sporadic kidney tumor

Age and sex

RCT is more frequent in males than in females, with a ratio of 3:2[7].

The older individuals possess the higher risk compared to young individuals. In fact, the peak incidence occurs in the sixth decade of life, with the majority (80%) of the cases occurring within the 40 to 69-year-old population. Although Wilms tumor is the most frequent renal tumor in the childhood, RCTs represents between 2% to 6% of the total renal tumors in infants, without differences between sexes [7].

Smoking

According with the International Agency for Research on Cancer (IARC), cigarette smoking is the most consistently established causal risk factor for RCC. Compared to never smokers, the risk augments about 50% in male and 20% in female smokers. A clear dose-response pattern of risk was observed with increasing amount of cigarettes smoked. Smoking cessation reduces the risk, although only among long-term quitters of ten or more years [11].

Obesity

Excess body weight has been established as a risk for RCC in several case-control and cohort studies, being estimated to account for over 40% of RCC, in the United States and over 30% in Europe. In prospective studies conducted worldwide, overweight and obese individuals at baseline were found to have elevated subsequent risks of renal cell cancer in a dose-response manner. In fact, each 5 kg/m2 body mass index (BMI) increase is estimated to enhance the risk in 24% and 34% for males and females,

respectively [12]. Nevertheless, the mechanisms by which obesity may increase RCC risk are not well understood [10].

Hypertension and use of anti-hypertensive medications

Hypertension or its treatment has been associated with the risk of RCC in several large prospective cohort studies [4]. Two studies measured blood pressure and observed an increased risk for RCC with elevated blood pressure in a clear dose-response association [13,14]. Users of diuretics and other anti-hypertensive medications were also indicated to elevate risk of renal cell cancer, but an independent effect from that of hypertension per se has not been established [11].

The biologic mechanisms underlying the association between hypertension and renal cell cancer are elusive, but are hypothesized to include chronic renal hypoxia and lipid peroxidation with formation of reactive oxygen species [11].

Acquired renal cystic disease

Patients on long-term hemodialysis and patients with end-stage renal disease (ESRD) have higher predisposition to develop Acquired renal cystic disease (ARCD). The incidence of RCC in ARCD patients is reported to be 3 to 6 times higher than in the general population. It was also reported that a larger period of dialysis may be associated with a higher incidence of RCC. Moreover, the incidence ratio, for both genders, is higher in patients with ESRD than in general population, and the age at RCC diagnosis is younger in ESRD patients than in the general population [4].

Occupation

RCC is not generally considered an occupationally associated tumor, but an excess risk for renal cell cancer has been associated in variety of occupations.
Trichloroethylene (TCE), considered a Group 2A “probable” human carcinogen by IARC, is certainly the most extensively chemically examined in relation to renal cell cancer risk. Epidemiologic evidence linking TCE to renal cell cancer is accumulating, with recent studies indicating a rising risk with increasing levels of exposure [11].

Surprisingly recent study suggests a possible association of renal cell cancer with TCE employment in agriculture, particularly among women [15]. Other agents such asbestos, polycyclic aromatic hydrocarbons, gasoline and other petroleum products have also been associated to increased RCC risk, but these associations are not fully proven [10].

1.2.2. Genetic factors

Von Hippel-lindau (VHL) syndrome

This syndrome is caused by germline mutations of *VHL* tumor suppressor gene, located on chromosome 3p25-26. Heritable clear cell RCC is caused by a germline mutation of one allele and an acquired mutation of the second allele[16]. These germline mutations are identified in nearly 100% of *VHL*-families. The VHL protein has an important role in cell cycle regulation and angiogenesis [17].

Clinical manifestations include, among others, the risk for developing RCC, pheochromocytoma, pancreatic cysts and retinal angiomas. The RCC is a carcinoma of the clear cell type and may be solid or cystic occurring at an average age of 37 years [18,19]. Inactivation of *VHL* is specific to clear cell RCC and is not found in other histological cell types of RCC[19]. Penetrance for each of these manifestations is incomplete, for example, RCC is only found in 40-50% of *VHL* mutation carriers [4].

Hereditary papillary renal cell carcinoma

Hereditary papillary RCC is an autosomal dominant trait [7], caused by activation of the *C-MET* proto-oncogene which maps the chromosome 7q31.1 and encodes a growth factor receptor. Mutations lead to constitutive activation of the receptor, which promotes tumor growth. Type 1 papillary RCC is exclusively observed in this syndrome, which specifically does not exhibit symptoms in other organs [4].

Hereditary leiomyomatosis renal cell carcinoma

Fumarate hydratase (*FH*) was identified as the gene responsible for hereditary leiomyomatosis renal cell carcinoma (HLRCC). Germinative mutations of *FH* gene have been detected in over 90% of North American individuals with hereditary leiomyomatosis renal cell carcinoma. The kidney tumors are mostly solitary and unilateral, although often display a mix of histologic patterns: papillary, tubulopapillary or solid [20].

Birt-Hogg-Dube Syndrome

The Birt-Hogg-Dub (*BHD*) gene maps to chromosome 17p11.2 and encodes the protein folliculin. This gene is also involved in sporadic RCC [7]. BHD is a syndrome in which patients develop cutaneous fibrofolliculomas, lung cysts, spontaneous pneumothoraced, and a variety of renal tumors, including chromophobe RCC, oncocytoma, and hybrid tumors that exhibit features of both of these entities[21]. However, other forms of RCC,

including clear cell RCC, have also been observed in this syndrome. Overall, penetrance for renal tumors is about 20% to 40%. Most renal tumors in BHD have limited biologic aggressiveness, although metastatic behavior and lethality have been already reported [4].

1.3. Pathology and classification

1.3.1 Histological subtypes

Current RCTs' histological classification was proposed in 2004 by the World Health Organization (WHO) [22]. This classification includes the achievements and contributions the prior classifications, in particular the Mainz (1986) and Heidelberg (1997) classifications [23,24] and describes categories and entities based on pathological and genetic analyses [25].

RCTs include a heterogeneous group of tumors with a spectrum from benign to malignant lesions. Malignant tumors are sub-classified into clear cell renal cell carcinoma (ccRCC), also called conventional carcinoma, papillary renal cell carcinoma (pRCC) and chromophobe renal cell carcinoma (chRCC). Benign tumors are subclassified into metanephric adenoma and adenofibroma, papillary renal cell adenoma, and renal oncocytoma [23].

RCTs are thought to arise from a multiplicity of specialized cells located along the length of the nephron. Both ccRCC and pRCC are thought to arise from the epithelium of the proximal tubule, whereas ChRCC, oncocytoma, and collecting duct RCC are considered to arise from the distal nephron, probably from the epithelium of the collecting tubule (figure 3). Each subtype has differences in genetics, biology and behavior [5].

Clear cell renal cell carcinoma

Clear cell renal cell carcinoma is the most frequent subtype of RCTs. It comprises more than 70% of all renal cell neoplasms [23].This is a malignant neoplasm composed of cells with clear or eosinophilic cytoplasm rich in glycogen and lipids [26]. Most of these tumors are solitary and occur with equal frequency in either kidney. Calcification, ossification and necrosis changes may occur. These tumors have a very vascular tumor stroma, regularly causing hemorrhagic areas. Some of them have a cystic appearance. This could be due to the necrosis (pseudo-cysts). Sometimes, ccRCC exhibit a pseudo-papillary architecture and often in these cases is difficult to distinguish them from pRCC [25].

Loss of chromosome 3p is a typical alteration in the vast majority of sporadic ccRCC[23]. These findings suggest the presence of one or more important tumor

suppressor genes (TSG) for RCC development on this chromosomal arm. The *VHL* tumor suppressor gene is one of the genes located at 3p and biallelic *VHL* inactivation is found in a high proportion of RCCs [27].

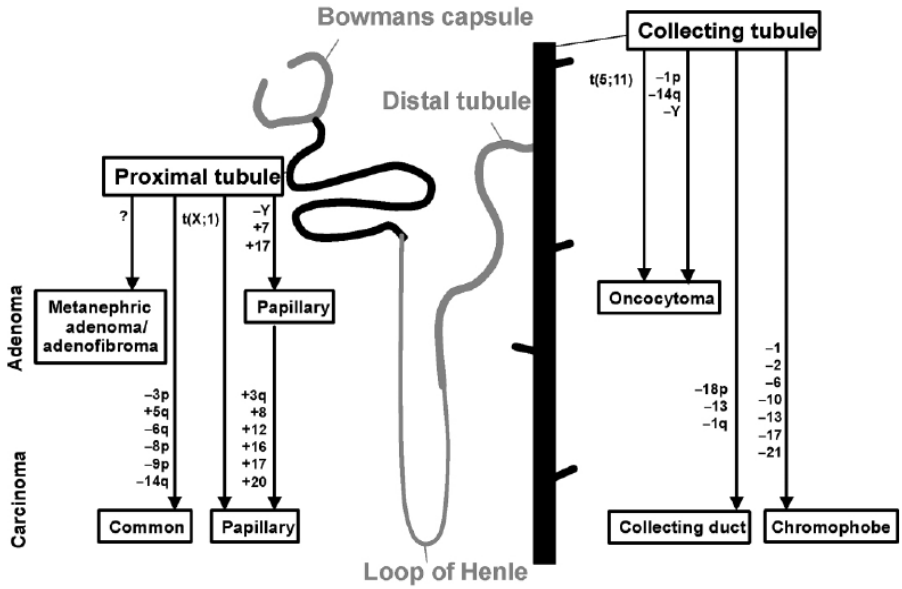

Figure 4: Classification of RCTs subtypes: The different RCTs subtypes are thought to originate from different parts of the renal tubular system: ccRCC/pRCC are supposed to arise of the proximal convoluted tubule and the chRCC/oncocytoma are supposed to arise of the collecting. *Adapted from* [27].

Papillary renal cell carcinoma

Papillary renal cell carcinoma is the second most prevalent subtype of RCTs, comprising approximately 10-15% of these tumors [28,29]. Papillary RCC has a variable proportion of papillae and could be multifocal or bilateral, normally containing necrosis, hemorrhage and cystic degeneration [25]. Histologically, it is characterized by epithelial cells forming tubules and papillae. The tumor papillae contain a fibrovascular core with aggregates of macrophages. Normally, psammoma bodies are also identifiable in this tumors [30]. Depending on the tumor cells morphology, pRCC is subclassified into two subtypes: Type 1 tumors with papillae covered by small cells with scarce cytoplasm and Type 2 tumor cells, regularly of higher nuclear grade with eosinophilic cytoplasm[16]. Trisomies or tetrasomies of chromosomes 7 and 17 and loss of the Y chromosome are the most commonly observed genetic changes in these tumors, often together with various combinations of additional trisomies in chromosomes 12, 16, and 20 [31].

Chromophobe renal cell carcinoma

Chromophobe renal cell carcinoma is the third most common RCT subtype. It represents approximately 5% of the total cases of RCC [25]. Macroscopically, chRCC is well-circumscribed solid neoplasm, and highly lobulated. The tumor size is larger than other subtypes [32]. Microscopically, they usually have a solid growth pattern, at times tubulocystic, with broad fibrotic septa [33]. Two types of tumor cells can be present. The first type is characterized by large and polygonal pale cells, with abundant transparent cytoplasm and prominent cell membranes. Usually, they are admixed with a second population of smaller cells with a eosinophilic and granular cytoplasm[33].

This tumor type is genetically characterized by losses of numerous chromosomes, mainly chromosomes 1, 2,10,13,17 and 21 have described in approximately 80%, 93%,93%,87%,90% and 70% of chRCC, respectively [33,34].

Oncocytoma

Oncocytoma is the most prevalent benign RCT (approximately 5%) [30]. It is non-capsulated, but well circumscribed. Histopathologically, this tumor has solid compact nests, acin tubules or microcystics. It is characterized by uniform round or polygonal cells. It has a densely granular eosinophilic cytoplasm. Necrosis and mitotic activity are rather uncommon in this tumor type [25,35].

Genetically, some cases show the translocation t(5;11)(q35,q13), whereas others display chromosome 1, 14 or Y loss [35].

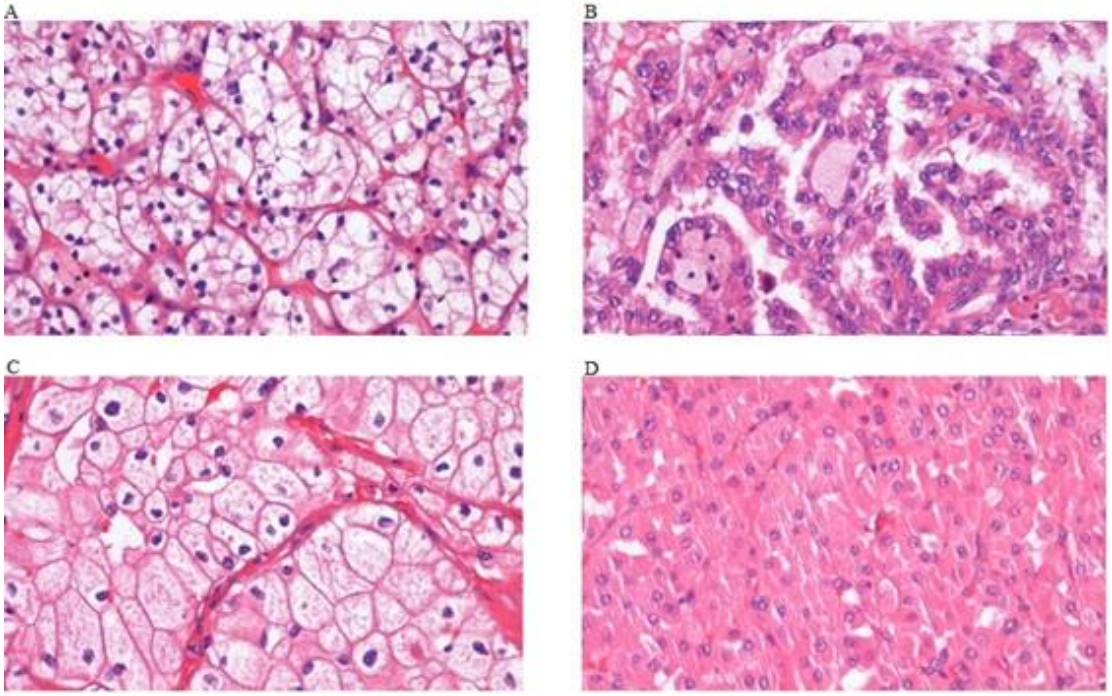

Figure 5: Morphologic characteristics among the RCTs subtypes. A- ccRCC; B- pRCC; C- chRCC and D: Oncocytoma.

1.3.2. Clinical and pathological staging

One of the most frequently used staging systems is the American Joint Committee on Cancer (AJCC) and International Union Against Cancer (IUAC) tumor-node-metastases (TNM) staging system. Herein, T represents the extension of the primary tumor, N refers to the lymph nodes status and M to distant metastasis. These criteria originate RCC grouping into four different categories [36,37]. Table 1 shows detailed information on TNM for Kidney cancer.

Table 1: Staging and stage grouping for RCC. *Adapted from [37].*

T-Primary tumor	
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
T1	Tumor 7 cm or less in greatest dimension, limited to the kidney.
T1a	Tumor 4 cm or less in greatest dimension, limited to the kidney.
T1b	Tumor more than 4 cm but not more than 7cm in greatest dimension, limited to the Kidney.
T2	Tumor more than 7 cm in greatest dimension, limited to the kidney.
T2a	Tumor more than 7 cm but less than or equal to 10 cm in greatest dimension, limited to the kidney.
T2b	Tumor more than 10 cm, limited to the kidney.
T3	Tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland.
T3a	and not beyond Gerota's fascia.
T3b	Tumor grossly extends into the renal vein or its segmental (muscle containing) branches, or tumor.
T3c	Invades perirenal and/or renal sinus fat but not beyond Gerota's fascia.
	Tumor grossly extends into vena cava below the diaphragm.
	Tumor grossly extends into vena cava above the diaphragm or invades the wall of the vena cava.
T4	T4 Tumor invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland)
	N – Regional Lymph Nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
	M – Distant metastasis
M0	No distant metastasis
M1	Distant metastasis
	Stage grouping
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T1 or T2 N1 M0 / T3 N0 or N1 M0
Stage IV	T4 Any N M0 / Any T Any N M1

1.3.3. Grading

Grading refers to how closely the cancer cells resemble normal kidney cells under the microscope. The Fuhrman system, described in 1982 by Fuhrman *et al*, is the most commonly used grading system in RCC [38]. This grading system is based on the nuclear size, irregularity and nucleolar prominence. Fuhrman grading system has a scale of 1 through 4 with grade 1 tumor being very little different from normal kidney cells and grade 4 tumors are quite different from normal kidney cells [38]. Currently, this grading system is the most important prognostic predictor in RCC, principally in clear renal cell carcinoma (ccRCC) type [25].

1.4. Therapy

Current treatment for RCC patients can be divided into local therapy and systemic therapy.

1.4.1. Localized treatment

Surgery is the only curative therapeutic approach for RCTs. For patients with T1 tumors or indications such as solitary kidney, bilateral renal tumor localization, renal insufficiency, as well as, hypertension, diabetes or hereditary renal cell carcinoma syndromes, the therapy of choice is currently partial nephrectomy. Contrarily, this approach is not suitable for patients with locally advanced tumor growth or unfavorable location. In these situations, the curative therapy remains radical nephrectomy either by laparoscopic or open surgery [16,39]. Radical nephrectomy is the recommended standard of care for patients with T2 tumors and smaller masses not treatable by partial nephrectomy, regardless of histological subtype [16,39]. Lymph node dissection does not appear to improve long term survival following nephrectomy [40].

Regarding surgical treatment of metastatic RCC, tumor nephrectomy is curative only if surgery can excise all tumor deposits. For most patients with metastastatic disease, tumor nephrectomy is palliative and complementary systemic treatments are necessary [39].

Radiotherapy may be useful for selected symptomatic patients with non-resectable brain or osseous metastases which do not respond to systemic treatment approaches [39].

1.4.2. Systemic therapy

Hormonal and chemotherapy are not standard treatment in RCC due to low response rates [16].

Systemic therapy has been rather ineffective to in metastatic renal cell carcinoma (mRCC). Chemotherapy as monotherapy is not recommended, at all. Opposite results have been accomplished with immunotherapy, namely interferon-α(IFN-α) has proven to increase survival over hormonal therapy [39]. Around 5% to 15% of cases of mRCC respond to IFN-α, however most of these responses are partial or short duration [16,41]. Interleukin-2(IL-2) has also been documented with response rates of approximately 7% to 27% [41].

Recent advances in molecular biology have led to the development of novel agents for treatment of mRCC, such as sunitinib, pazopanib, bevacizumab, temsirolimos, everolimus. These drugs have shown efficacy in phase III trials, and were recently approved by the Food and Drug Administration (FDA) for application in RCC patients [16,39]. After treatment with these targeting agents, a substantial improvement of progression-free and overall survival has been achieved [39].

1.5. Tumor prognosis

There has been a gradual improvement in RCC prognosis over the last decades, with 5-year relative survival rates as high as 64% in 2002, compared with less than 40% in the early 1960s [16].

Factors influencing prognosis can be classified into anatomical, histological, clinical, and molecular. Anatomical factors are usually included in the TNM staging classification system [39]. According to this staging system, the 5 year cancer-specific survival rates ranged from 97% for pT1A to 20% for pT4. Presence of lymph node metastases predicts a poor prognosis, with cancer-specific survival rates of 5–30% and 0–5% at 5 and 10 years, respectively. Metastases to other organs (lung, bone, brain) are associated with survival rates of 50%, 5-30% and 0-5% at 1,5 and 10 years, respectively [16].

The histological variables mainly include the Tumor grade and RCC subtype. Concerning the tumor grade, grades 1/2 have slow growth and spread and tend to have a good prognosis, whereas the 3/4 have a worse prognosis. However, grading of tumor cells is an important factor in assessing prognosis, mostly in ccRCC [42]. In univariate analysis, there is a trend of better prognosis for patients with chRCC when compared with pRCC and ccRCC. However, in multivariate analysis, TNM stage, Fuhrman grade and the performance status, but not histological classification, are preserved as independent prognostic variables. Tumor necrosis is also a strong independent predictor of poor

outcome for ccRCC but not for chrRCC or pRCC. Microvascular invasion and presence of sarcomatoid features are also reported as adverse prognostic indicators [16].

Clinical factors consist of patient performance status, localized symptoms, anemia and platelet count.

Finally, and concerning the molecular markers investigatedto date, none of these markers has provided evidence to improve the predictive accuracy of existing prognostic systems [39].

The clinical behavior of RCC depends on complex interactions between several prognostic factors, nonetheless the most useful predictors of patient outcome, currently available, comprise patient performance status, tumor stage and grade [16].

1.6. Symptoms and diagnosis

Renal tumors are asymptomatic and non-palpable in early stages of disease. More than 50% of renal tumors are detected incidentally by imaging examinations to investigate non-specific symptoms or diseases [43]. The standard presentation of RCC includes the classic triad of hematuria, pain and a flank mass, but more than 40% of RCC patients do no exhibit these three symptoms. In fact, the only 10% of RCC patients whom present any symptom often have advanced disease at diagnosis [5,44].

Because histological subtypes differ in clinical aggressiveness and prognosis [45,46], accurate classification is required for appropriate patient management. Moreover, most RCTs are clinically silent at their earliest stages, and 20-30% are diagnosed when metastatic spread has already occurred [47]. The widespread use of imaging techniques (mainly ultrasonography) has increased the detection of suspicious renal masses, prompting new pre-operative diagnostic challenges as histological diagnosis using needle biopsy material, however this appoach meets important limitations, hampering an accurate categorization in many instances [48]. Renal tumors biopsy is increasingly being used in diagnosis before ablative and systemic therapy. Biopsy aims to determine malignancy, subtype and grade of the renal mass. In most series, a core biopsy demonstrates high specificity and high sensitivity for the presence of malignancy, although 10–20% of biopsies are inconclusive [39]. In this setting, diagnosis relies mainly on morphologic features, some of which are shared by tumor subtypes [49]. The discrimination between chRCC (mainly the eosinophilic variant) and oncocytoma is one of the most critical, in which differential diagnosis might be difficult. However, chRCC is a malignant neoplasm, capable of local invasion and metastatic spread, whereas oncocytoma is a benign tumor, therefore requiring two different management strategies.

1.7. The problem distinguishing Oncocytoma from Chromophobe Renal Cell Carcinoma

As mentioned above, the most important and challenging differential diagnosis in renal neoplasms are between oncocytoma and chRCC particularly its eosinophilic variant with the abundant granular eosinophilic cytoplasm. This might be due to the fact that they have a common origin in nephron, specifically in the collecting duct [50]. Importantly, they should be correctly diagnosed because both tumors have different behaviors and clinical outcomes (oncocytoma is a benign tumor, whereas chRCC is a malignant tumor with metastatic potential).

Over the years, several attempts have been made to assist morphology in the differential diagnosis between chRCC from oncocytoma, including immunohistochemical profiles [47,48,51], histochemical stains [52] and gene expression analysis [53]. However, sensitivity and specificity of those techniques are suboptimal and prompt the need for more accurate biomarkers, although, the results of these studies are inconsistent [53].

Concerning morphological features, the presence of fibrous capsule and its thickness has been described as a feature to distinguish chRCC and oncocytoma, however, a large number of cases are needed to support this finding [50].

Immunohistochemical markers had also been tested to distinguish chRCC and oncocytoma, namely cytokeratin 7 , claudin 7 and parvalbumin, which were described as preferentially expressed in chRCC over oncocytoma [47,48], S100 protein and CD82, which were found to be preferentially expressed in oncocytoma [48,51]. More recently, Lee *et al* described caveolin-1 and MOC-31, which show a preferentially expression in chRCC and not in oncocytoma [53]. Nonetheless, the results of these studies are inconsistent and need to be further confirmed by additional studies [48,54,55].

Similarly, some reports have suggested that Hale´s colloidal iron stain as a helpful method for the differential diagnosis of chRCC from oncocytoma. Nevertheless, this stain is technically challenging and the results are quite variable, and low reproducibility [50,52].

Moreover, gene expression studies have been also performed in order to find a gene/ panel of genes amenable to distinguish this two subtypes of RCTs . Specifically, T-cell differentiation protein 2 (*MAL2*), prominin 2 (*PROM2*), protease, serine, 8 (*PRSS8*), epithelial splicing regulatory protein 1 (*FLJ20171*) and adaptor-related protein complex 1, Mu 2 subunit (*AP1M2*) gene's expression was evaluated. These genes showed a differential expression between chRCC and oncocytomas [54]. However, the clinical diagnostic usefulness of these markers lacks of validation.

Interestingly, some recent studies have attempted to discriminate RCTs subtypes using epigenetic alterations as will be later detailed.

2. Epigenetics

The term “Epigenetics” was first used by C. H. Waddington in 1939 [56]. It derives from the word “epigenesis”, and it was first introduced to describe “the causal interactions between genes and their products, which bring the phenotype into being” [57], thus relating genes and development even though the Watson-Crick Deoxyribonucleic Acid (DNA) structure had not yet been reported. During almost half a century, the word epigenetics was used in this sense [57]. About forty- three years later, in 1996, Riggs and colleagues defined epignetics as: “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” [58]. As used nowadays the term “Epigenetics” comprehends both heritable and transient changes in gene expression that do not involve a change in primary DNA sequence [58,59]. Epigenetic mechanisms that control gene expression can be divided into three main categories: DNA methylation, post-translational histone modifications, and expression of non-coding RNAs [60]. Together these different mechanisms generate the so called “epigenome” which is defined as: “Act to regulate what genetic information can be accessed by the transcription and translation machinery” [61]. When epigenetic patterns are deregulated it might induce abnormal activation or inhibition of different pathways which can lead to a disease , such as occurs in cancer [62].

2.1.1. DNA Methylation

DNA methylation is perhaps the most widely studied epigenetic mechanism [63]. This modification is found in genome of almost all organisms, and the patterns and levels are variable across species [64,65].

DNA Methylation, involve the addition of methyl group to the fifth carbon position of a cytosine, originating in the formation of a new base- 5-methylcytosine (m5C). This reaction is catalyzed by DNA methyltransferases (DNMTs), using S-adenosyl-L-methionine (SAM) as a donor of methyl groups [66,67]. So far, two different classes of DNMTs have been described: The maintenance and *de novo* methyltransferases. The most important maintenance is DNMT1, and its major function happens during DNA replication, and has preferential activity for hemi-methylated DNA. Whereas *de novo* methyltransferases include DNMT3A and DNMT3B, which preferentially methylate previous unmethylated Cytosine-Phosphate-Guanine (CpG) sequences, but are capable to methylate hemi-methylated DNA [65,68]. DNA methylation is associated with gene

silencing expression. Globally, there are two alternative mechanisms by which DNA methylation inhibits gene expression. This may occur through the obstruction of transcriptional activators on methylated regions within or near the promoter, and / or indirectly through by recruiting methyl-CpG-binding domain proteins (MBDPs) that induce chromatin changes. This family of proteins use transcriptional co-repressor molecules to silence transcription and to modify surrounding chromatin, providing a link between DNA methylation and chromatin remodeling [68,69].

In mammals, DNA methylation occur mostly in cytosines that precede guanines, these are called dinucleotide CpGs [56]. CpG dinucleotides, are not distributed across the human genome but are instead concentrated in short CpG-rich DNA stretches called “CpG islands”, in which CpG´s concentration is higher than elsewhere in the genome. CpG islands are preferentially located at the 5´end of genes [61,70]. In fact, they occupy about 60% of the human gene promoters [63].

Generally, the CpG islands are not methylated in normal cells and a few percent (~6%) become methylated in tissue-specific manner during early development or differentiated tissues, which results in stable silencing of the associated promoter, for instance in genomic imprinting and X-chromosome inactivation [64,66]. Furthermore, alterations in DNA methylation ate linked to many human diseases, including cancer [69].

2.1.2. Histone modifications

Histones are the chief protein components of chromatin, which are composed with by an octamer of histones (two H2A-H2B dimers and one H3-H4 tetramer) to form a nucleosome, which are the fundamental unit of chromatin [71], and presents of a globular C-terminal domain and a flexible unstructured N- terminal tail [72]. The N-terminal tail of histones can undergo a variety of post-translational covalent modifications of specific residues, namely: methylation, acetylation, ubiquitylation, sumoylation and phosphorylation [73]. From these, methylation and acetylation are the most studied, and the best characterized. These modifications are known to play a critical role in chromatin packaging and key cellular processes, such as replication, transcription, and DNA repair [71]. Acetylation is catalyzed by histone acetyltransferases (HATs) and can be reversed by the enzymatic action of histone deacetylases (HDACs). In general, acetylation of lysine residues decreases the affinity of histones for DNA creating an “open” chromatin conformation that allows gene transcription [74].

Histone methylation-modifying enzymes include histone methyltransferases (HMTs) and histone demethylases (HDMs), which present high substrate specificity [71]. Contrarily to acetylation, histone methylation can lead either to transcriptional activation or repression depending upon which residues are modified and the type of modifications present. For

example, methylation of lysines 4, 36, and 79 of histone 3 (H3K4me3, H3K36me, and H3K79me) is associated with transcriptionally active regions, whereas methylation of lysines 9 and 27 of histone 3 (H3K9 and H3K27) and of lysine 20 of histone 4 (H4K20) is commonly linked to heterochromatin formation and the presence of inactive promoters [75]. Genome-wide histone modifications have also been described in malignant cells. However, the current knowledge concerning the involvement of histone modifications in tumorigenesis is less clear than DNA methylation modifications. These two different epigenetics phenomena are thought to be inter-dependent [56,76].

2.1.3. Non-coding RNAs

Non-coding RNAs (ncRNAs) are RNAs that despite not encoding proteins are biologically functional [77,78]. Recently, ncRNas have been implicated in different molecular, cellular and organismal events in eukaryotic cells. It been established to play a role in transcription regulation through their interaction with several transcription factors, and also interacts with chromatin-modifying enzymes demonstrating its importance in maintaining a proper chromatin conformation [79,80]. Other variety of complex mechanisms such as gene silencing, DNA imprinting, and RNA interference have already been connected to theirs function [81]. Therefore, changes in ncRNAs expression levels have been associated in development of many different human diseases, including a cancer [77,78]. NcRNAs comprise several different classes, according to their length and their function. MicroRNAs (miRNAs), transcribed ultraconserved regions (T-UCRs), small nuclear RNAs (snoRNAs), PIW-interacting RNA (piRNAs), large intergenic non-coding RNAs (lincRNAs), and the heterogeneous group of long non-coding RNAs (lncRNAs) [77,78]. Nevertheless, the functional relevance of the non-coding genome in normal development and physiology and for disease have been particularly evident for miRNAs [78].

2.2. MicroRNAs Biogenesis and Mode of Action

MiRNAs have been the most widely investigated class of ncRNAs. Constitutes a group of endogenous single-stranded non-coding small RNA of approximately 18-22 nucleotides in length [78,82]. The first miRNA to be identified was *link-4*, in 1993, by Victor Ambros and colleagues, from a study of developmental timing in the nematode C*. elegans* [83].

Since then, hundreds of miRNAs have been identified and currently, more than 2000 human miRNAs are registered in miRBase database.

The biogenesis of miRNA begins in the nucleus, and the miRNAs are transcribed by an RNA polymerase II into a capped and polyadenylated precursor with hairpin structures, called pri-miRNA [84,85]. Following transcription, a pri-miRNA forms a stem-loop structure with a double-stranded RNA (dsRNA) of approximately 33 nucleotides (Figure 2-a) [86].

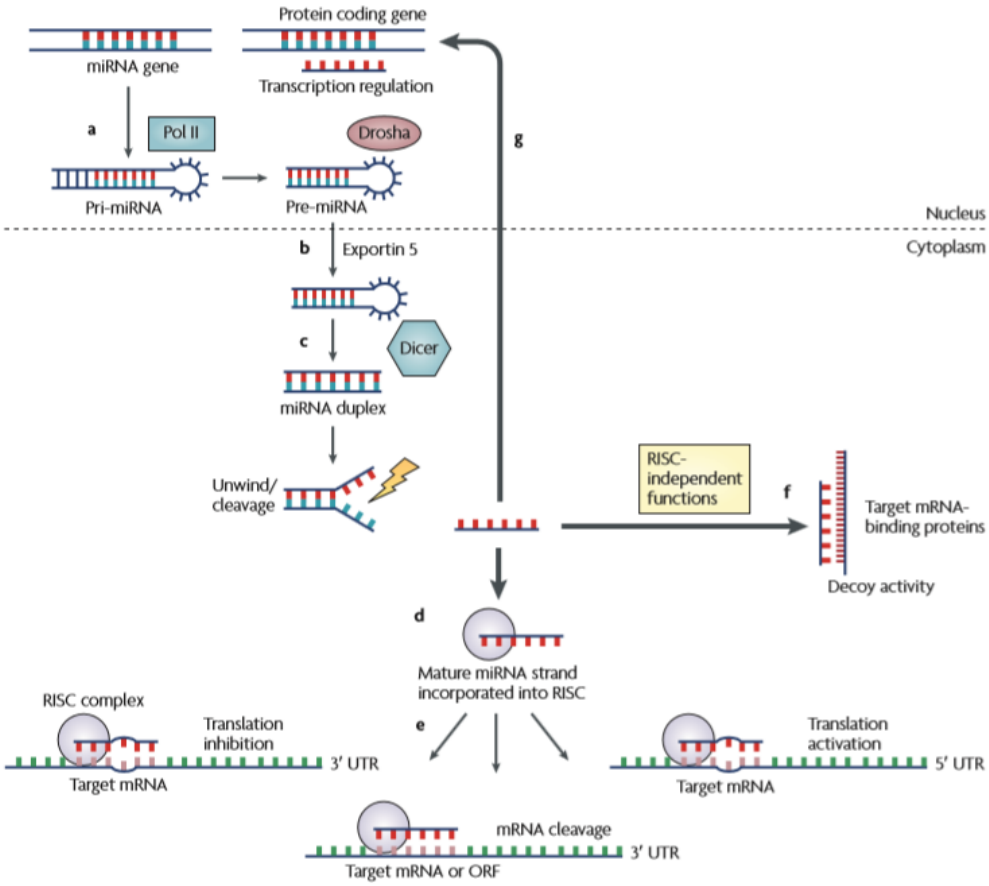

Figure 6: miRNA biogenesis and mechanism of action. *Adapted from* [90]

Pri-miRNA are recognized and cleaved in the nucleus by a complex known as microprocessor which is composed of Rnase II Drosha and Di George syndrome critical region 8 (DGCR8). The cleavage produces a hairpin RNA of about 65 nucleotides that is called a precursor-miRNA (pre-miRNA) [86]. Surprisingly, Drosha mediated processing of pri-miRNA into pre-miRNA is not obligatory, alternatively some miRNAs (named mirtrons) which are located in short introns of host genes, bypass the Drosha processing step and the pre-miRNA are derived as a byproduct of splicing event. After splicing, the excised intron is debranched and trimmed by lariat-debranching enzyme to generate the pre-miRNA [78,87]. At this moment, the mirtron pathway merges with the canonical miRNA pathway[88] and the harpin pre-miRNA is then exported from the nucleus to cytoplasm, by

exportin-5(XPO5) in complex with Ran-GTP, and is further processed by another RNase enzyme called Dicer, which acts in complex with the dsRNA-binding protein Trans-activator RNA-binding protein 2, (TARBP2) [87,89]. The cleavage results into an imperfect RNA (dsRNA), duplex-designated miRNA, witch named miRNA/miRNA*, with approximately for 19-24 nucleotides [90,91].Only one strand of the miRNA duplex(mature miRNA), preferentially in 5´ segment more loosely paired with the opposite strand is selected to function as a mature mirRNA , and are incorporate into a large protein complex (Argonaute protein) to form the RNA-induced silencing complex (RISC), whereas the passenger strand is subjected to degradation [82,92]. RISC leads the regulation of gene expression at post-transcriptional level, binding mostly through partial complementarity to a sequence in the target mRNA generally located at the 3'-untranslated region (3'UTR) [84,93].

Depending on level of complementarity between miRNA and its target mRNA sequence, this regulation is performed by two main ways: if perfect or near-perfect complementarity is established, the RISC induces mRNA degradation; if they exhibit an imperfect pairing, mRNA translation into a protein is blocked [87,94](Figure 2). Although the most frequent site of interaction is the 3´ UTR region of the target mRNA, recent studies have described that miRNAs could also bind to the open reading frame sequence (ORF) sequences, as well as the 5´UTR. This interaction has been associated with activation rather than repression [90,95]. Interestingly, a specific miRNA may regulate multiple mRNAs in the same manner that a single mRNA may be targeted by multiple miRNAs [92,93]. Additionally, miRNAs can also regulate gene transcription by binding directly or by modulating methylation patterns at target gene promoter. Furthermore, miRNA can also bind directly to proteins, in a seed sequence in a RISC-independent manner, interfering with their RNA-binding functions [90,96]. While still poorly documented, it has been described that miRNAs are able to regulate gene expression at transcriptional level by direct binding to the DNA [97,98]. In mammalian cells, it is estimated that miRNAs control the activity of more than 60% of protein-coding genes and they play crucial roles in the regulation of multiple pathways and cellular processes, including proliferation, differentiation, apoptosis, development and metabolism [78,92,99]. Due to their involvement in mostly all biological mechanisms, the abnormal expression of miRNAs contributes to a range of human diseases, including cancer [78,88].

MiRNA genes can be located within coding mRNAs (40% are intronic or exonic) or in the intergenic regions. About one-third of miRNAs are organized in clusters, and these may affect several miRNAs altering thousands of protein targets[100].

2.3. MicroRNAs and cancer

Cancer is a complex genetic disease caused by the accumulation of mutations, which lead to deregulation of gene expression and uncontrolled cell proliferation. Given the wide impact of miRNA on gene expression, it is not surprising that a large number of altered miRNAs have been connected in cancer [84].

The first evidence linking aberrant miRNA expression and human cancer, was found in B-cells leukemia in 2002 by Calin e colleagues [101]. Actually, altered expression of miRNAs are considered a common characteristic of all human tumors, and they can initiate carcinogenesis or drive a tumor progression [100].

Altered expression of miRNAs in cancer have been associated to different mechanisms (Figure 7) [102]. Similarly to protein-coding genes, more than half miRNA genes are located in chromosomal regions that frequently exhibit amplification, deletion or translocation [103-105]. Mutations and single nucleotide polymorphisms (SNPs) within miRNAs have also been also described in different types of cancer [106,107].

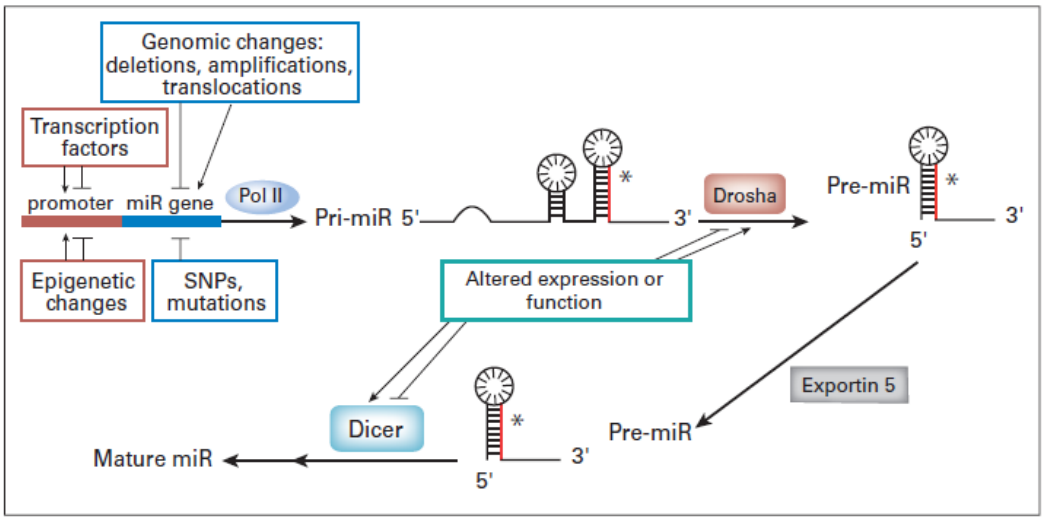

Figure 7: Mechanisms of miRNA dysregulation. *Adapted from* [102]

Epigenetics changes such as DNA methylation can also lead to microRNAs' expression deregulation in cancer. An extensive analysis of genome sequenced for miRNA genes have shown that approximately half of them are associated with CpG islands, suggesting that they could be a target of this mechanisms' regulation [87]. Conversely, methylation is not the only epigenetic mechanism that might affect miRNA expression, since it has been shown that histone deacetylase inhibition (HDAC) inhibitors have the potential to promote downregulation of some miRNAs [99]. Furthermore, miRNA deregulation can result from increased or decreased transcription activity of transcription factors at the promotor. In addition to genetic and epigenetic alterations, miRNA expression can also be modulated by defects in the machinery involved in miRNA

biogenesis [87]. Indeed, changes in miRNA levels might be a consequence of genetic alterations in different molecules involved in miRNA processing, such as Drosha [88], XPO5[108], Dicer [109], and TARBP2 [110]. These alterations affect the production of the pri-miRNA, their processing to mature miRNA form and/or interaction with mRNA targets, and have been already implicated in neoplastic transformation [78].

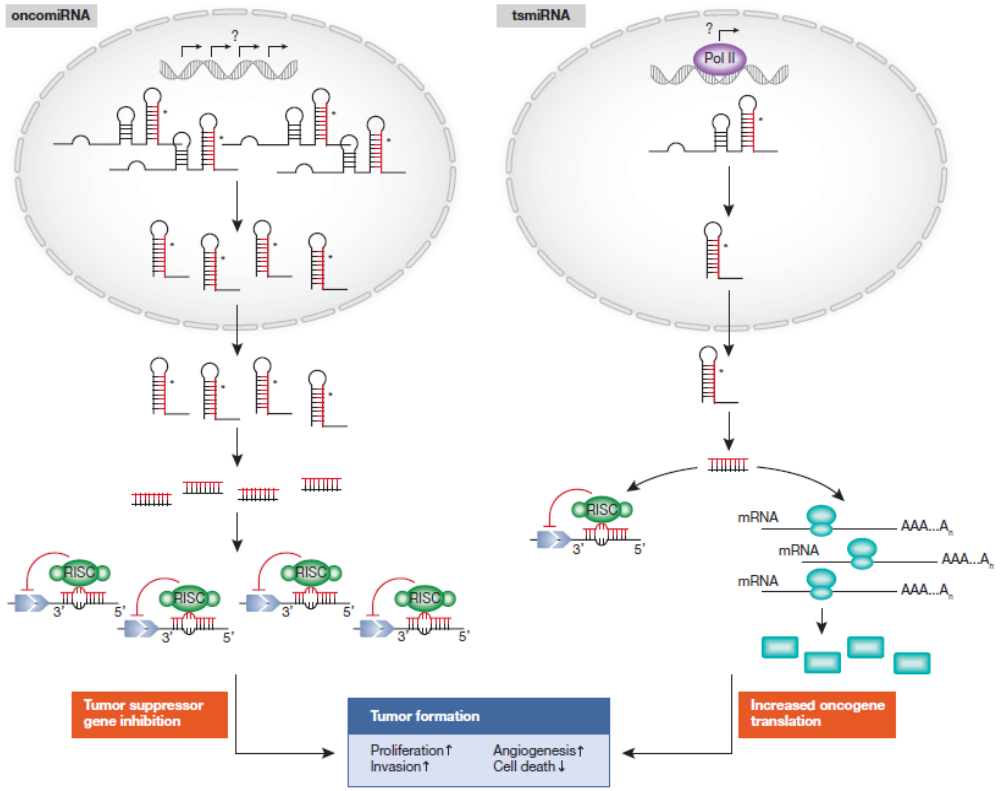

Figure 8: MicroRNAS as oncogenes or tumor suppressor genes. *Adapted from* [87]

MiRNAs have been suggested to function as oncogene or tumor suppressor based on their inhibition of tumor-supressive or oncogenic target mRNAs [87,99] (Figure 8). MiRNA with oncogenic activities are named *oncomirs.* This term indicates the miRNA are constitutively overexpressed and promote tumor cell growth by inhibing tumor suppressor genes or genes that control cell cycle progression, differentiation or apoptosis. By contrast, tumor suppressor miRNAS, *anti-oncomir* usually prevent tumor development by inhibiting oncogenes [111].

Furthermore, tumor suppressor miRNAs could undergo loss of function in tumors due to chromosomal rearrangements, deletions or mutations [92]. Although it is known that both overexpression and depletion of specific miRNAs play pathogenic roles in tumor progression, most human tumors are characterized by a general defect in miRNA production that results in global downregulation of miRNA expression [112]. Interestingly,

some miRNAs may have dual functions, and act both as tumor suppressors and oncogenes, depending on the context [93].

2.4. MicroRNAs as tumor biomarker

Recent studies have demonstrated that different tumors and tumor subtypes have specific miRNA signatures which may be useful as diagnostic and/or prognostic markers [86,91]. MiRNA signatures can distinguish not only between normal and tumor tissues and identify tissues origin, but also they can discriminate different subtypes of a particular cancer, or even specific oncogenic abnormalities [87]. An important issue in clinics is clearly represented by the need of biomarkers for an early diagnosis, which are particularly important considering that survival and prognosis of patients depends on the stage of the tumor at the time of detection, with an early diagnosis usually been associated with best prognosis. MiRNAs have shown a great promise as new early diagnosis biomarkers, for example in study published by Du Rieu and colleagues in 2010 [87,113]. Lu and colleges showed that miRNA expression profiles indicate that miRNAs are a better indicator for discriminate cancer tissues from normal tissues and can successfully classify even poorly differentiated tumors [112]. Furthermore, miRNAs can function as truthful molecular markers because they are relatively stable (more stable than mRNA) and resistant to RNase degradation, probably due to their small size. [87]. MiRNAs can be isolate from different types of biological material, such formalin-fixed paraffin-embedded (FFPE) specimens [114], from fresh frozen samples [115], and from different biological fluids, such as blood (total blood, plasma or serum) [43,116] and urine [117]. Additionally to the expression profile studies based on microarray platforms, other methods for analyzing microRNAs have been developed, as quantitative real-time polymerase chain reaction (RT-qPCR) [49] and *in situ* hybridization [118].

2.5. MicroRNAs deregulated in kidney cancer.

Deregulation of miRNA expression seems to be critical for RCC development and progression [119]. Indeed, several miRNA have been found to be deregulated in RCTs, although most of the studies have studied mainly on ccRCC. Some of them were found upregulated (miR-16 [120,121];miR-18a [122] ;miR-20a [122];miR-21 [76,121,122]; miR-34a and miR-34-b [76,121]; miR-92a [122]; miR-155 [76,119-121], miR-185 [76,121]; miR-210 [76,121,122]; miR-224 [76,120] and let-7 [121,122], whereas others were found downregulated (miR-125b [76,121]; miR-141 [76]; miR-133b [76]; miR-200b [76]; miR-200c [76]; miR-429 [76,121]; miR-506 [121,123]; miR-508-3p [121,123]; miR-509-5p [123];

miR-509-3-5p [121,123]; miR-510 [76,121] and miR-541 [76,121,123]. These miRNAs are the most consistently reported alterations.

Only a limited number of studies addressed the potential use of miRNAs as RCC detection biomarkers. In this regard, differential miRNA expression patterns in neoplastic and non-neoplastic renal tissues, as well as among different renal tumor subtypes have been documented. The discrimination between ccRCC and normal kidney tissue might be accomplished by a panel of nine miRs (miR- 21,miR-34a,miR-142-3p,miR-155,miR-185,miR-200c,miR-210, miR-224, and miR-592) [115]; a combination of miR- 141 and miR-155 [120] or through the differential expression of miR-92a, miR-210, and miR-200c [119]. Moreover, regarding miRNAs signatures for each RCT subtypes, analysis of miRNA microarray data showed that tumors derived from the proximal nephron (ccRCC and pRCC type I) and tumors derived from the distal nephron (oncocytomas and chRCC) can be distinguished by their miRNA profile (Valera et al., 2011), supporting previous observations for ccRCC and chRCC by Nakada and colleagues [124]. These differential expression patterns of microRNAs might be also used to subclassify RCTs [49,125,126]. Indeed, Petillo and colleagues showed that ccRCC and pRCC were differentially expressed in 27 miRNAs, with ccRCC expressing higher levls of miR-203 and miR-424 compared to pRCC, whereas pRCC expressed higher levels of miR-31 and mir-504 than ccRCC. Additionally, similar miRNA expression patterns was reported for chRCCs and oncocytomas.[126]. In a different study by Valera and colleagues miR-143, miR-19a, miR-21, miR-29a, miR-181a, and miR-378 were found to be only expressed by chRCC, whereas mir-146a was preferentially expressed in oncocytoma [119]. Additionally, chRCC showed higher levels of miR-203, mir-200b, miR-197, and miR-320 than oncocytoma, whereas miR-186 was overexpressed in oncocytoma comparing with chRCC [49,126]. However, most of these studies only performed microarray analysis in a limited number of samples, and usually not representing all histological subtypes. Finally, two recent studies investigated miRNA expression levels in RCC patients' serum and identified miR-1233 and mi-210 as promising biomarker for RCC detection and monitoring [43,116].

Altered levels of miRNAs might also provide prognostic information in ccRCC. Interestingly, it was previously showed that miR-155 and miR-21's overexpression correlates with tumor size [122]. Moreover, in a different study, higher levels of miR-210 were found in tumors displaying the highest Fuhrman grade [119]. Furthermore, overexpression of miR-32 as well as of miR-210, miR-21, let-7i, and miR-18a correlated with poor survival [122,126,127]. Additionally lower miR-106b levels were associated with metastatic disease and poorer relapse-free survival [128].

Aims of the study

In two recent reviews [129,130], we found that five microRNAs (miR-21, miR-141, miR-155, miR-183 and miR-200b) had been suggested to display diagnostic or prognostic value in RCT [49,115,120,124,126]. Thus, we aimed to confirm and extend those findings through expression analysis of a five miRNA panel in a single series of RCT, comprising the four major subtypes.

Specifically, the main objectives of this thesis were:

- Evaluate the expression of a panel miRNAs in fresh-frozen tissue to:
    - Distinguish tumor and normal tissue.
    - Distinguish different histological subtypes.
    - Determine its diagnostic value.
    - Determine its prognostic value.

- Assess the two best performance miRNAs in *ex vivo* aspirate biopsy samples from kidney cancer patients.

Material and methods

1. Clinical samples

A total of 130 fresh frozen tissues were prospectively collected and included in this study, including 120 renal cell tumors, comprising 30 cases of each of the four major subtypes (ccRCC, pRCC, chRCC, and Oncocytoma), and 10 normal tissues. Additionally, 60 cases of *ex vivo* biopsies (15 of each subtype) were included for further validation. These samples were selected from patients diagnosed and treated at the Portuguese Oncology Institute – Porto (Portugal), between 2003 and 2007, who underwent partial or total nephrectomy, after informed consent. Normal tissues were obtained from morphologic normal kidney tissue of patients that were subjected to nephrectomy due to urothelial carcinoma. *Ex vivo* biopsies were obtained during surgery and tumor tissue samples were immediately snap-frozen, stored at -80ºC and subsequently cut in a cryostat for RNA extraction.

Fuhrman grade and TNM stage of all cases of RCC were previously assessed by histological slides from formalin-fixed paraffin embedded tissues fragments from the same surgical specimens. Relevant clinical data was collected from clinical charts. This study, as well as the use of samples and the access to clinical data, was approved by the Institutional Review Board (Comissão de Ética) of Portuguese Oncology Institute – Porto, Portugal.

2. RNA extraction

Total RNA was extracted from fresh frozen tissues and *ex vivo* biopsies using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Briefly, 1500µL of Trizol® Reagent was added to each 2.0mL tube and tissues were homogenized using a rotor-shaker. Tubes were incubated for 5 to 10 min at room temperature and then 300µL of Chloroform (Merck, Germany) were added. In biopsies samples the protocol was similarly performed, but the quantitative Trizol® Reagent and chloroform were 500µL and 200µL, respectively. Tubes were vigorously hand-shacked for 15sec and incubated for 3min at room temperature, followed by a 15min 12,000g centrifugation at 4ºC. Then, the upper phase was collected. RNA was purified using the PureLinkTM RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer's indications. RNA concentration and purity ratios were then evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Aditionally, RNA quality was checked by electrophoresis in a 2% agarose gel.

3. Reverse transcription

Reverse transcription (RT) was performed using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT human pool A (Applied Biosystems, Foster City, CA, USA), from total RNA samples (Figure 9). The reaction mix had a final volume of 12µL and included 3µLof total RNA (750ng), 1.6µL of megaplex RT primers (10x), 0.4µL of dNTPs with dTTP (100 mM), 3µL of multiScrible reverse transcriptase (50 U/µL), 1.6µL of 10x RT buffer, 0.2µL of RNase inhibitor (20 U/µL) and 0.4µL of nuclease-free water. Reactions were incubated in PCR tubes followed by 40 cycles at 16°C for 2min, 42°C for 1min and 50°C for 1sec, before 85°C for 5min.

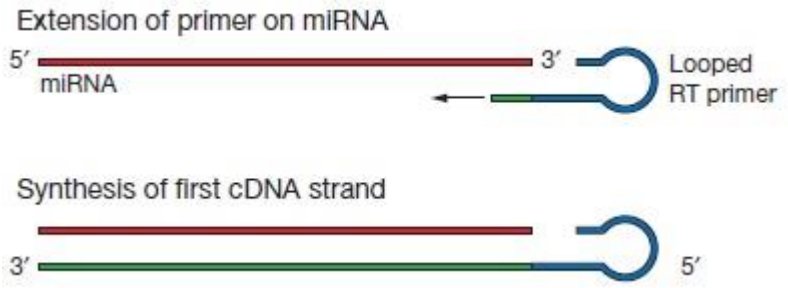

Figure 9: Reverse transcription reaction. *Adapted from Megaplex™ Pools For microRNA Expression Analysis*

4. Quantitative real time RT-PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed using TaqMan® Small RNA Assays (Applied Biosystems, Foster City, CA, USA), in a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol.). A TaqMan® MGB probe contains a reporter dye linked to the 5'-end and a minor groove binder (MGB) at the 3'-end. Besides MGB, there is also a non-fluorescent quencher (NFQ) at the 3'-end of the probe. During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 10 I). The DNA polymerase cleaves only probes that are hybridized to the target separating the reporter dye from the quencher dye and the separation results in increased fluorescence by the reporter (Figure 10 II). This fluorescence is read by Real-Time PCR System and it is proportional to the amount of PCR product.

Briefly, for each well was added 0.5µL of TaqMan Small RNA Assay (20x), 0.75µL of RT product, 5µL of TaqMan Universal PCR Master Mix II no UNG (2x), and 3.75µL of nuclease-free water. According to manufacturer's instructions, protocol conditions were:

50°C for 2 min, 95°C for 10min, followed by 40 cycles at 95°C for 15sec and 60°C for 1min. Expression of five selected miRNAs were assessed, using taqMan® microRNA assays: hsa-miR-21 (RT00397), hsa-miR-141 (RT000463), hsa-miR155 (RT002623), has-miR-183 (RT002269) and hsa-miR-200b (RT002251). All samples were run in triplicate and two water blanks were added to each plate as negative controls.

Results from the qRT-PCR were analyzed using the 7500 Software version 2.0.5 (Applied Biosystems, Foster City, CA, USA). Levels of miRNAs expression were determined using the relative standard curve method, which provides more accurate quantitative results in comparison with the ΔΔCt method [131].

In each sample, the mean quantity of each miRNA was normalized to the mean quantity for the endogenous controls RNU48 (RT001006) and RNU6B (RT001093), according to the following formula:

$$\text{miRNA expression} = \frac{\text{miRNA mean quantity}}{(\text{RNU48 quantity mean} + \text{RNU6B quantity mean})/2}$$

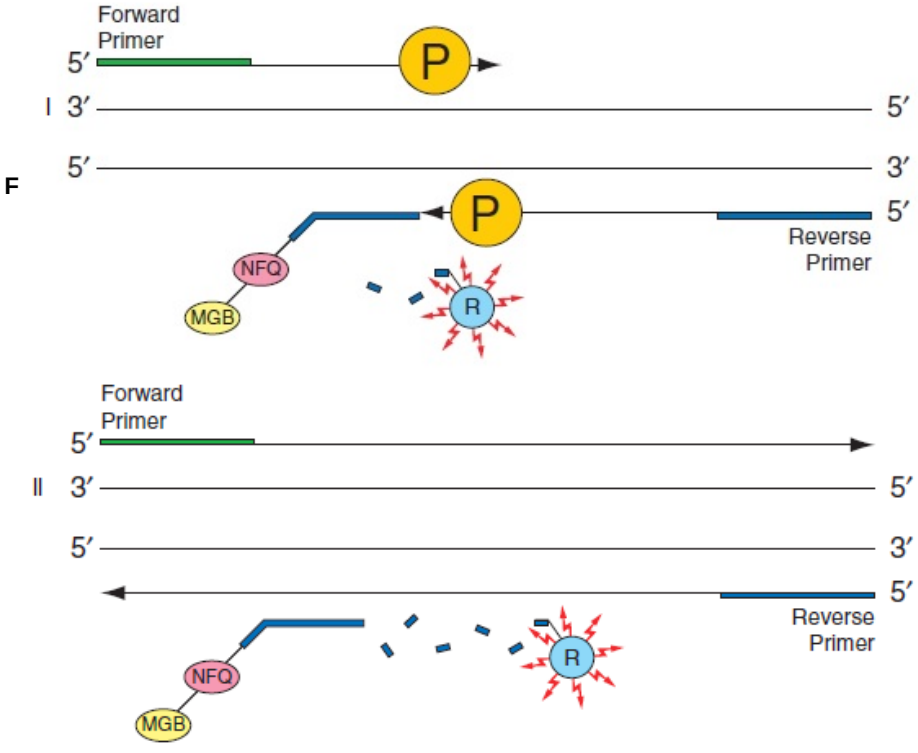

Figure 10: Quantitative reverse transcription-PCR reaction. TaqMan® technology chemistry. MGB – minor groove binder; NFQ – non- fluorescent quencher; R – reporter; P – DNA polymerase. *Adapted from TaqMan® Small RNA Assays protocol.*

5. Statistical analysis

Differences in expression levels of the related miRNAs between the different histological subtypes were firstly analyzed using non-parametric Kruskal-Wallis test, followed by pairwise comparisons using non-parametric Mann-Whitney U-test. The relationship between miRNAs expression and clinico-patological variables (gender, pathological stage, Fuhrman grade) was evaluated using Mann-Whitney U-test. A Spearman Nonparametric Correlation Test was additionally carried out to ascertain correlation values between age and miRNAs expression levels. A Receiver Operator Characteristics (ROC) curve was created by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity). In order to perform a panel of two microRNAs, we used logistic regression, and the areas under the curve (AUC) were calculated. Disease-specific survival curves (Kaplan-Meier with log-rank test) were computed for standard variables such as histological subtype, pathological stage, Fuhrman grade, age, gender and also miRNA expression levels. A Cox-Regression model comprising all significant variables and the Fuhrman grade (multivariate test) was computed to assess the relative contribution of each variable to the follow-up status. In statistical analyses, pathological stage and Fuhrman grade was recode into groups (pT1-pT2 versus pT3- pT4 and grade 1-2 versus 3-4, respectively).

Statistical analysis was performed using SPSS for Windows, version 20.0 [SPSS, Chicago, IL, USA], and considered statistically significant when $p < 0.05$, and when comparing multiple groups p value was adjusted with the Bonferroni method, thus significance value was set at $p < 0.017$.

Results

Clinical Samples

Relevant clinical and pathological features of the patients included in this study are summarized in Table 2. No significant differences in age or gender between RCTs patients and normal tissue donors were apparent.

Table 2: Clinical and pathological features of patients included in this study, including the data for the two sets of samples (Fresh frozen tissues and *ex-vivo* biopsies)

	Fresh frozen tissues		***Ex-vivo* aspiration biopsies**
	Tumor	**Normal**	**Tumor**
Number of patients, n	120	10	60
Age, median (range)	62 (30-84)	65 (20-83)	60 (30-82)
Gender, n (%)			
Male	71 (59.2)	7 (70.0)	35 (58.3)
Female	49 (40.8)	3 (30.0)	25 (41.7)
Histological subtype, n (%)		n.a.	
Clear cell RCC	30 (25.0)		15 (25.0)
Papillary RCC	30 (25.0)		15 (25.0)
Chromophobe RCC	30 (25.0)		15 (25.0)
Oncocytoma	30 (25.0)		15 (25.0)
Pathological stage, n (%)		n.a.	
pT1	46 (38.3)		25(41.7)
pT2	19 (15.9)		8(13.3)
pT3	24 (20.0)		12(20.0)
pT4	1 (0.8)		-
N.A. (oncocytoma)	30 (25.0)		15(25.0)
Furhman grade, n (%)		n.a.	
1	3(2.5)		0 (0.0)
2	27(22.5)		12 (20.0)
3	44 (36.7)		20 (33.3)
4	16 (13.3)		12 (20.0)
n.a.	30 (25.0)		16 (26.7)

MicroRNA expression levels and clinicopathological correlates

The relative expression levels of miR-21, miR-141, miR-155, miR-183 and miR-200b were determined in fresh frozen tissues of 120 RCTs and 10 normal renal tissue samples. No statistically significant associations were disclosed between miRs expression levels and any of the clinicopathological characteristics (age, gender, Fuhrman grade categories or pathological stage). RCTs showed significantly lower expression levels of miR-21, miR-141, and miR-200b compared with normal tissues (p < 0.001 for all; Figure 11A and Supplementary Table 1). Moreover, expression levels of all target miRs differed significantly between benign and malignant RCTs. Oncocytomas displayed lower expression levels for all tested miRs except miR-183 (Figure 11B and Supplementary Table 2).

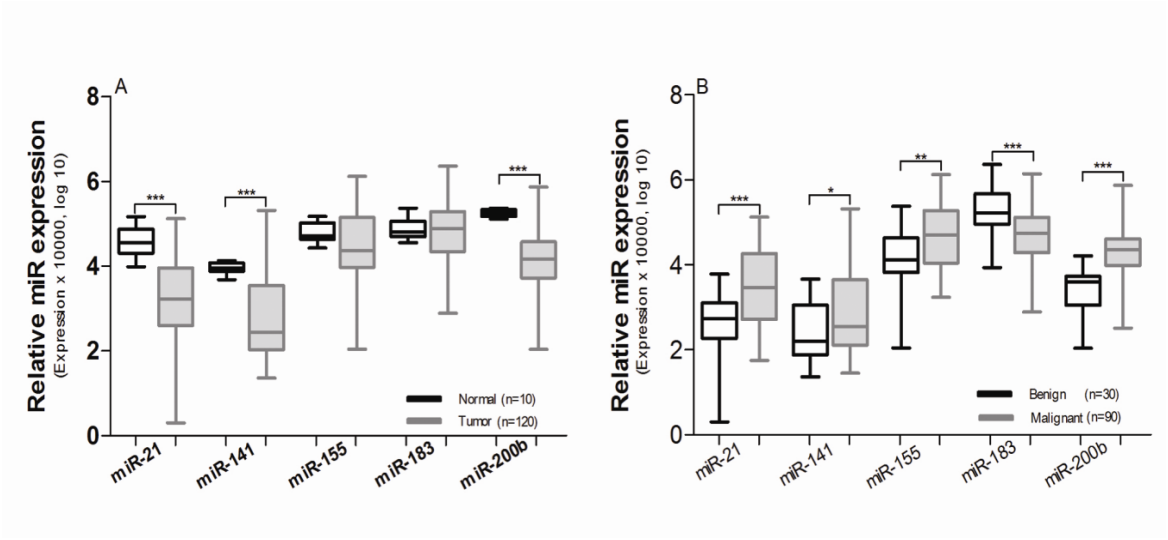

Figure 11: Distribution of microRNAs expression levels in kidney tissues. A: normal *vs*. tumor tissues. B: benign *vs.* malignant tumor tissues. Statistically significant differences are represented with three asterisks (p<0.001), two asterisks (p <0.01) and one asterisk (p<0.02).

Expression levels of all miRs differed significantly among the four RCT subtypes (p < 0.001 for all, Kruskall-Wallis test; Table 3). Pair-wise comparisons are shown in Table 4 and graphically illustrated in Figure 12.

Generally, oncocytomas displayed the lower miR expression levels, significantly differing from pRCC or ccRCC in four miRs (miR21, miR-155, miR-183 and miR-200b), and from chRCC in two miRs (miR141 and miR-200b). Interestingly, ccRCC and pRCC only differed for miR-155 expression levels whereas chRCC differed from ccRCC and pRCC for miR-21, miR-141 and miR-155 expression levels. In addition miR-183 expression levels were also different between chRCC and ccRCC Table 4. Thus, reduced expression of miR-200b surfaced as the most discriminative feature between oncocytomas and RCCs.

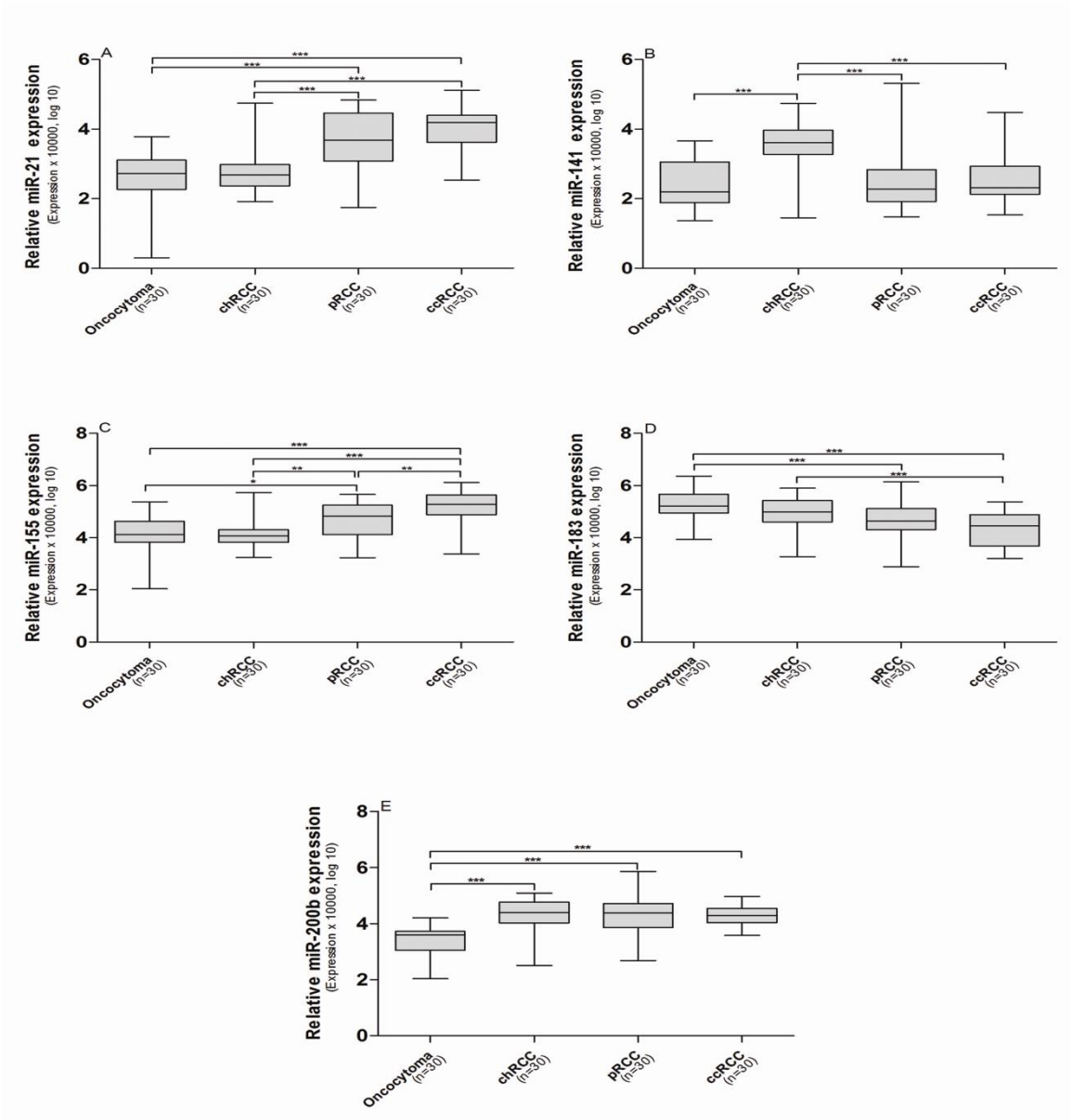

Figure 12: Distribution expression levels of miR-21, miR-141, miR-155, miR-183 and miR-200b according with the histological subtype of RCTs. Statistically significant differences are represented with three asterisks ($p<0.001$), two asterisks ($p< 0.003$) and one asterisk ($p<0.0125$).

Table 3: Distribution of microRNAs expression levels among different histological subtypes in fresh-frozen tissues.

	Oncocytoma	chRCC	pRCC	ccRCC	p-value, K-W
miR-21	5.3 (0.02-60.9)	4.0 (0.8-560.2)	47.9 (0.6-689.3)	155.5 (3.5-1325.8)	<0.001
miR-141	7.9 (0.2-45.9)	83.5 (0.3-552.2)	76.8 (0.3-2063.3)	25.75.7 (0.3-301.2)	<0.001
miR-155	374.9 (1.1-233.7)	339.6 (14.5-5340.1)	1054 (17.1-4595.9)	3148.8 (23.74-13299)	<0.001
miR-183	5034.7 (87.1-23207.1)	1690.3 (18.8-8013.8)	1350.1 (7.7-13865)	512.5 (15.9-2360.7)	<0.001
miR-200b	0.3 (1.1-161)	367.9 (3.3-1244)	611.6 (4.8-7445.1)	249.1 (38.1-930.2)	<0.001

K-W, Kruskall-Wallis test; ccRCC, clear cell renal cell carcinoma; pRCC, papillary RCC; chRCC, chromophobe RCC

Table 4: Comparison of microRNA´s expression among renal cell tumor subtypes in fresh-frozen tissues.

	p-value*, M-W test				
	miR-21	**miR-141**	**miR-155**	**miR183**	**miR-200b**
Oncotytoma vs. ccRCC	<0.001	n.s.	<0.001	<0.001	<0.001
Oncocytoma vs pRCC	<0.001	n.s.	0.012	<0.001	<0.001
Oncotytoma vs. chRCC	n.s.	0.001	n.s.	n.s.	0.001
pRCC vs. ccRCC	n.s	n.s.	0.003	n.s.	n.s.
ccRCC vs. chRCC	<0.001	0.002	<0.001	<0.001	n.s.
pRCC vs. chRCC	<0.001	<0.001	0.002	n.s.	n.s.

*Statistically significant when p<0.0125, Bonferroni's correction; M-W, Mann-Whitney test; ccRCC, clear cell renal cell carcinoma; pRCC, papillary RCC; chRCC, chromophobe RCC; n.s., not significant.

Diagnostic performance of microRNAs expression levels in tissue samples

The performance of the five target miRs was assessed in three different settings: identification of RCTs (*vs.* normal renal tissue), discrimination of malignant from benign tumors, and distinction of chRCC from oncocytoma. For these purpose, the cutoff value was the highest value obtained by the ROC curve analyses (sensitivity + (1-specificity)). Validity and information estimates for each marker and for the best combination of markers are displayed in Table 5. ROC curve analysis showed that a panel comprising expression of miR-141 and miR-200b allowed for the discrimination between RCT and normal renal tissue with 99.2% sensitivity and 100% specificity, corresponding to an AUC of 0.991.

Table 5: Validity estimates for each tested miR and for the best combination of miRs in each diagnostic setting, in fresh-frozen tissues

		miR-21	miR-141	miR-155	miR-183	miR-200b	miR-141/miR-200b
RCT *vs.* NRT	SE	76.7	81.7	–	–	97.5	99.2
	SP	100	100	–	–	100	100
	PPV	100	100	–	–	100	100
	NPV	26	31	–	–	77	90.9
	Accuracy	78	83	–	–	98	99.2
	AUC	89.9	89.7	–	–	98.7	99.1
RCC *vs.* Onc	SE	48.9	25.6	50.0	72.2	96.7	85.6
	SP	93.3	100	83.3	73.3	90.0	100
	PPV	95.7	100	90.0	89.0	96.7	100
	NPV	37.8	13.0	35.2	46.8	67.5	69.8
	Accuracy	60.0	33.0	58.3	72.5	95.0	89.2
	AUC	75.9	64.9	66.7	75.1	91.4	91.4
chRCC *vs.* Onc	SE	–	76.7	–	–	83.3	90.0
	SP	–	86.7	–	–	90.0	100
	PPV	–	85.2	–	–	89.3	100
	NPV	–	78.7	–	–	84.4	90.9
	Accuracy	–	81.6	–	–	86.7	95.0
	AUC	–	81.9		–	89.6	90.0

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC, area under the curve; RCT, renal cell tumor; RCC, renal cell carcinoma; chRCC: chromophobe RCC: NRT: normal renal tissue; Onc: oncocytoma

Moreover, the same panel allowed for the differentiation between benign and malignant tumors with 85.6% sensitivity and 100% specificity, displaying an AUC of 0.912. Furthermore, expression levels of miR-141 and miR-200b also distinguished chRCC from oncocytoma with 90% sensitivity and 100% specificity, corresponding to an AUC of 0.90 (Figure 13 A-B).

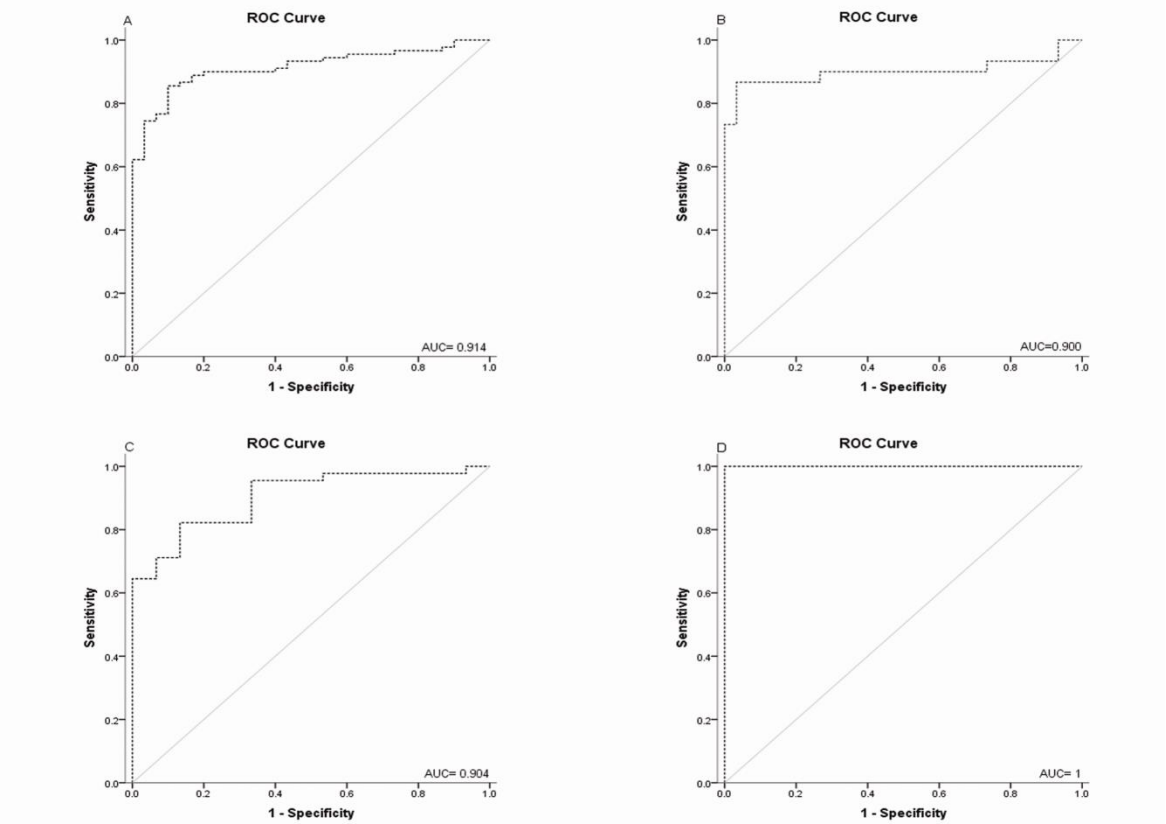

Figure 13: Receiver operator characteristic (ROC) curves evaluating the performance of the gene panel (miR-141 and miR-200b): (A, C) as a biomarker for malignant renal tumors (B, D) as a biomarker of chRCC. A and B were performed in kidney tissue samples; C and D in ex vivo aspiration renal biopsies

Survival analysis

The median follow-up of this series of RCT patients was 65 months (range: 1-120 months). A total of 12 patients (13.3%) have died from RCC during this period. Disease-specific survival (DSS) analysis showed that tumor subtype ccRCC or pRCC and higher pathological tumor stage (pT3-T4) were significantly associated with worse outcome (p = 0.011 and $p < 0.001$, respectively; Figure 14A-B).

Although age at diagnosis over 62 years was associated with worse DSS (p=0.035), gender and Fuhrman grade did not disclose any prognostic value within the available follow-up time. Concerning microRNAs expression levels, miR-200b and miR-183 did not display any prognostic value. However, high expression levels of miR-21 and miR-155 and low expression levels of miR-141 were associated with worse DSS (p = 0.006, p = 0.037 and p = 0.024, respectively; Figure 14C-E). However, in multivariate analysis only pathological stage independently predicted prognosis, whereas miR expression levels did not retain independent prognostic value (Supplementary Table 3).

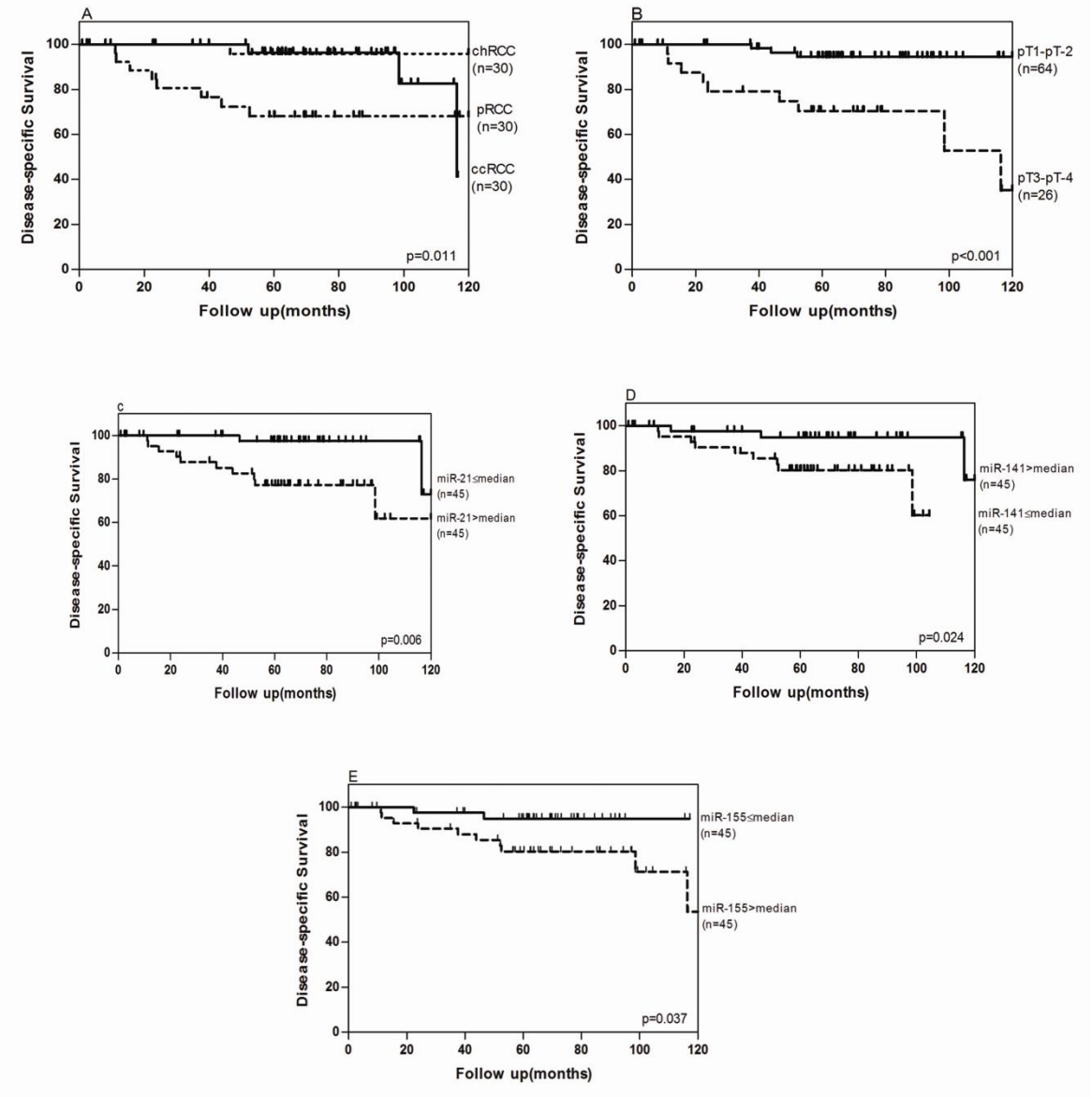

Figure 14: Disease-specific survival according to pathological and molecular parameters. (A) histopathological classification; (B) pathological stage; (C-E) miR expression levels.

Validation of the microRNA panel in *ex-vivo* aspiration biopsies

The two best performing miRNA in tissue samples, miR-141 and miR-200b, were then selected for analysis in *ex vivo* aspiration biopsies. This set comprised 60 *ex vivo* fine-needle aspiration biopsies. Relevant clinical and histopathological data are summarized in Table 2 and the relative expression levels for each miR are depicted in Supplementary Table 4.

The expression of these two microRNAs in *ex-vivo* aspiration biopsies follows the same trend observed in fresh frozen tissues. Not only when we compared the expression of microRNAs between malignant and benign tumors (Figure 15) , but also when comparing the different histological subtypes (Figure 16).

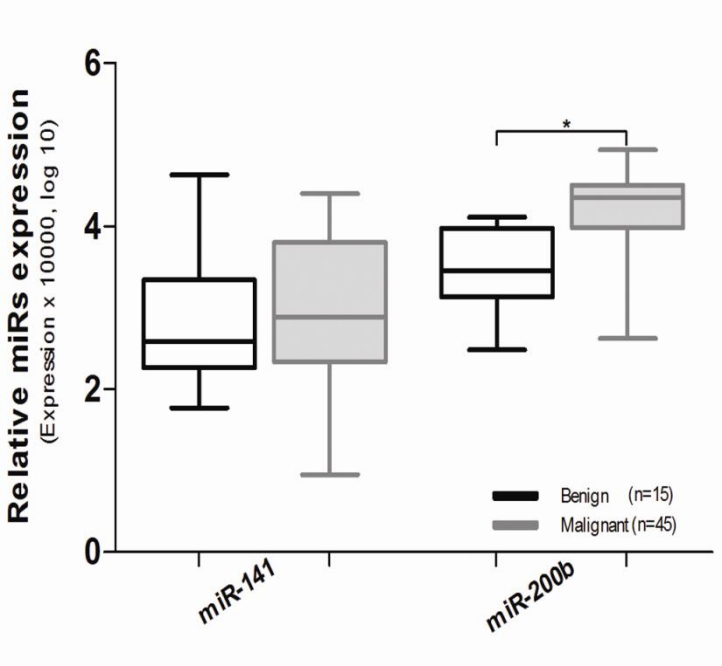

Figure 15: Distribution of miR-141 and miR-200b expression levels in benign and malignant tumors of ex vivo kidney aspiration biopsies. Statistically significant differences are represented with one asterisks ($p<0.001$).

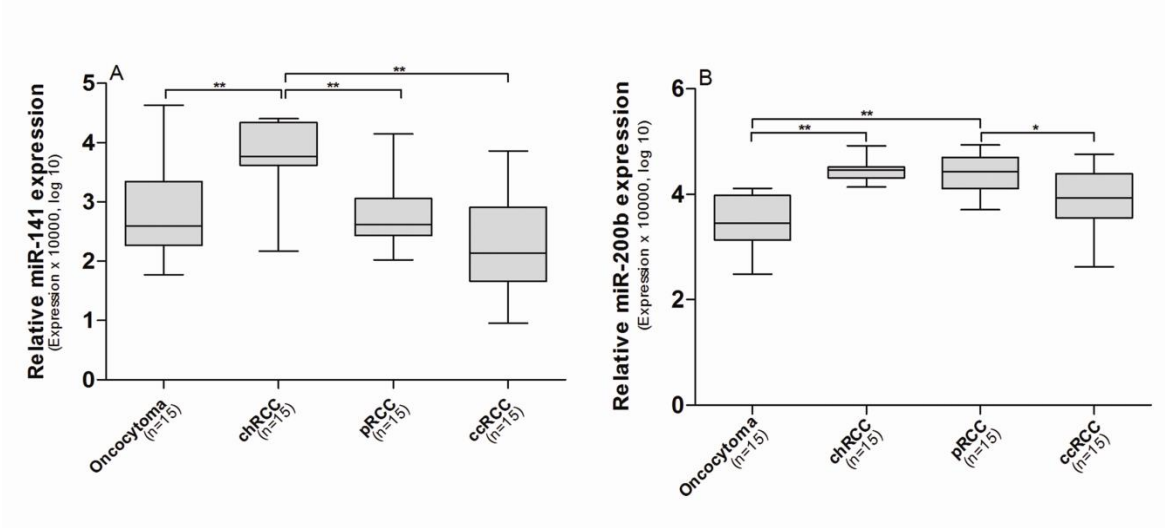

Figure 16: Distribution of miR-141 and miR-200b expression levels according with the histological subtype of RCTs in ex vivo kidney aspiration biopsies. Statistically significant differences are represented with one asterisk ($p<0.005$) and two asterisks ($p<0.001$).

Remarkably, expression levels of this panel of microRNAs was able not only distinguish benign from malignant RCT with 73.3% sensitivity and a 100% specificity (AUC of 90.4%), but also oncocytoma from chRCC with 100% sensitivity and 100% specificity (AUC of 100%) (Figure 13C-D; Table 6).

Table 6: Validity estimates for each tested miR and for the best combination of miRs in each diagnostic setting, in *ex vivo* aspiration biopsies

	(%)	miR-141	miR-200b	miR-141/miR-200b
RCC *vs.* oncocytoma	SE	35.5	73.3	73.3
	SP	93.3	93.3	100
	PPV	94.1	97.1	100
	NPV	34.5	53.8	55.0
	Accuracy	50.0	78.3	80.0
	AUC	57.5	88.4	90.4
chRCC *vs.* oncocytoma	SE	73.3	100	100
	SP	93.3	100	100
	PPV	91.6	100	100
	NPV	77.7	100	100
	Accuracy	83.3	100	100
	AUC	84.4	100	100

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC, area under the curve; RCC, renal cell carcinoma; chRCC: chromophobe RCC

Discussion

In this study we aimed to define a small set of miRs that might allow for accurate identification of RCTs, as well as for discrimination between oncocytoma and RCCs, especially chRCC. The definition of such panel would be of clinical relevance as diagnostic workup of suspicious renal masses incidentally found by abdominal ultrasonography is increasingly more frequent and demanding. Indeed, each RCT subtype displays quite dissimilar clinical behavior, ranging from totally benign to overtly malignant and successful pre-therapeutic cytological or histological assessment is limited [45,46]. Only a few studies addressed the use of miRNAs expression as biomarkers for RCTs detection, and these have been mainly restricted to the ccRCC subtype, or have analyzed only a very limited number of samples [49,115,116,120,126,132]. After an extensive review of published literature, we selected five miRNAs (miR-21, miR-141, miR-155, miR-183 and miR-200b) with putative diagnostic and/or prognostic value [129,130], and tested them in a relatively large set of tissue samples that comprised the major histological subtypes. To ascertain their clinical and pathological relevance, a validation study was subsequently performed in a set of *ex vivo* fine-needle aspiration biopsies.

Of the five miRs tested, three (miR-21, miR-141 and miR-200b) were significantly downregulated in RCTs compared to normal renal tissue, whereas the remainder did not disclose statistically significant differences. In three previous reports, miR-21 was found to be upregulated in RCT [114,115,127], which apparently contradicts our results. However, in those studies normal renal tissue was obtained from nephrectomy specimens harboring RCT, which did not occur in our study as normal renal tissue was collected from kidneys harboring urothelial tumors of the renal pelvis. This is an important issue as we have previously showed that morphologically normal renal tissue from kidneys harboring RCT display epigenetic alterations in line with the respective tumors [133]. Remarkably, variations in miR-21 expression among RCT subtypes observed in our study matches that reported by Faragalla and co-workers [114], with ccRCC depicting the highest median levels, followed by pRCC, chRCC and oncocytoma. Indeed, only miR-21 expression levels of “normal renal tissue” are notably different between our results and their study

[114]. These findings prompt the need for an adequate definition of “normal tissue” as the interpretation of results in tumors might be considerably biased.

Concerning miR-141, our results corroborate those of two previous reports [124,125], in which the highest expression levels of miR-141 were observed in chRCC and these were significantly different from all the other RCT subtypes. Thus, high miR-141 expression levels seem to be a hallmark of chRCC and might constitute a valuable biomarker for discrimination from oncocytoma. Strikingly, a miR profiling of ccRCC, also identified miR-141 (and 200b) as being downregulated in ccRCC, although with concurrent upregulation of miR-155 [120]. These results are in line with ours as we found that the highest miR-155 expression levels in ccRCC and pRCC, significantly differing from those of oncocytoma and chRCC. Our findings concerning miR-200b mirror those of Youssef *et al.* [49], which observed increased expression in chRCC compared to oncocytoma, although in a smaller dataset. Interestingly, in our larger dataset we were able to demonstrate that miR-200b expression levels were significantly lower in oncocytomas, compared to all RCC subtypes. Overall, the comparisons of miRs expression levels among RCT subtypes also denote the common origin (segment of the nephron) of ccRCC and pRCC, on one hand, and of chRCC and oncocytoma, on the other hand, emphasizing the importance of searching for discriminative biomarkers, which might enable accurate identification of each RCT subtype.

Once the main purpose of this study was the identification of small panel of biomarkers based on specific miRs expression levels, we chose the two miRs that jointly might allow for accurate discrimination between normal kidney and RCTs as well as that of oncocytomas from RCC, in general, and chRCC, in particular. A panel comprising miR-141 and miR-200b demonstrated the best performance in frozen-tissue samples, displaying AUC values ranging from 90.0 to 99.1. Although these results are interesting *per se*, its clinical usefulness depends on the possibility of using it in diagnostic samples. For that purpose, we further validated this biomarker panel in a set of fine-needle aspiration biopsies performed *ex vivo*. Although this procedure is not completely

equivalent to an imaging-guided diagnostic fine-needle aspiration biopsy performed in a patient (which may yield lower amounts of tumor cells), it is, nonetheless, the best approximation without jeopardizing patients' diagnosis. On the other hand, because the nephrectomy specimen is already available, its histopathological characterization is guaranteed whereas diagnostic biopsies may not be followed by surgical excision, thus precluding accurate tumor classification for comparison purposes. Remarkably, the biomarker panel performance in *ex vivo* biopsies was comparable to that of fresh-frozen tissues. To the best of our knowledge, this is the first attempt to demonstrate the feasibility of using miRs as tumor biomarkers in renal tumor biopsies, and may thus constitute a significant step forward in the development of epigenetic-based biomarkers for management of RCC suspects.

The clinical significance of our findings could be extended if miRs expression levels might convey prognostic information. Thus, we performed disease-specific survival analysis using expression levels determined in fresh-frozen tissue samples. As expected, tumor subtype and pathological stage were of prognostic value in univariate analysis, although only the later showed independent prognostic value in multivariate analysis. Remarkably, miR-21, miR-141 and miR-155 expression levels also displayed prognostic significance in RCC, although only in univariate analysis. A possible explanation for these findings may lie in the association between specific miRs expression levels and tumor subtypes. Indeed, whereas for miR-21 and miR-155 the association with poorer DSS was observed for higher (> median) expression levels, the opposite was verified for miR-141. Interestingly, higher miR-21 and miR-155 and lower miR-141 expression levels were associated with pRCC and ccRCC subtypes, which displayed the worse prognosis, compared to chRCC. The fact that tumor subtype did not surfaced as independent prognostic parameter for DSS in multivariate analysis is most likely due to the association between tumor subtype and pathological stage, as pT3-4 tumors were mostly of pRCC or ccRCC subtype. Our findings concerning miR-21 and miR-141 are corroborated by previous reports, although with generally smaller patient cohorts [114,120,127]. In

addition, the prognostic value of miR-155 expression levels has been reported for breast cancer [134] and non-small cell lung cancer [135,136], whereas miR-21 and miR-141 expression seem to be of prognostic significance in non-small cell lung cancer [135,136] and colon cancer [137], respectively.

The aforementioned association of specific miRs altered expression and RCT subtype might also provide clues concerning the cause of miRs dysregulation. It is widely acknowledged that RCT subtypes display characteristic chromosomal aberrations, including whole or partial deletions and/or duplications [16]. Strikingly, some of those alterations might explain the altered pattern of miRs expression. For instance, miR-200b is mapped at 1p36.33 and loss of 1p or of the whole chromosome 1 is frequently observed in oncocytoma and chRCC. On the other hand, miR-21 and miR-155 are mapped at 17q23.1 and 21q21.2-21.3, which are frequently lost chromosomal regions in chRCC. Conversely, pRCC, which commonly show gain of chromosome 17, are among the RCT subtypes with higher miR-21 expression levels. However, other variations in miRs expression might not be explained by chromosomal-level alterations and the respective cause(s) remain to be investigated.

Conclusion

Herein, we demonstrate that expression levels of a panel of two miRNAs (miR-141/miR200b) allows for accurate distinction of normal kidney from RCT tissue samples, as well as for accurate discrimination among RCT subtypes, including the separation of benign from malignant RCT. Furthermore, the selected miR panel is able to convey prognostic information, although not independent of tumor subtype or pathological stage. Importantly, the same panel displays an impressive performance for accurate detection of RCC in clinical samples obtained from *ex vivo* fine-needle aspiration biopsies, demonstrating the feasibility of this approach in routine diagnostic practice.

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Supplementary

Supplementary Table 1 : Expression levels of microRNAs in fresh-frozen tissues from normal kidney and renal cell tumors.

	Normal	Tumor	P-Value (M-W)
miR-21 (med, min-max)	357.0 (97.9-1477.9)	106.4 (0.02-1325.1)	<0.001
miR-141 (med, min-max)	93.4 (47.9-134.9)	48.5 (0.2-2026.3)	<0.001
miR-155 (med, min-max)	688.5 (17.2-13298.9)	1229.5 (1.1-13299.0)	0.231
miR-183 (med, min-max)	873.7 (361.5-2360.9)	1897.8 (7.7-23207.1)	0.993
miR-200b (med, min-max)	1820.8 (1317.2-2336.9)	319.3 (1.1-7445)	<0.001

M-W, Mann-Whitney test; med, median value; min, minimum value;max, maximum value

Supplementary Table 2: Expression levels of microRNAs in fresh-frozen tissues from benign (oncocytoma) and malignant (clear cell, papillary and chromophobe carcinomas) renal cell tumors

	Benign tumors	Malignant tumors	P-Value (M-W)
miR-21	5.3 (0.02-60.9)	9.7 (0.6-1325.8)	<0.001
miR-141	7.9 (0.23-45.9)	62.0 (0.3-2063.3)	0.015
miR-155	374.6 (1.10-233.7)	1538.6 (7.1-13299)	0.006
miR-183	5034.7 (87.0-23207.1)	1184.3 (7.2-1385)	<0.001
miR-200b	40.3 (1.09-161)	230.0 (3.3-7445.1)	<0.001

M-W, Mann-Whitney test

Supplementary Table 3: Cox regression models assessing the potential of clinical variables and miR´s in the prediction of disease-specific survival for 90 patients with renal cell carcinoma.

		HR (95% CI for HR)
miR-21	Histological subtype	
	ccRCC	0.55 (0.03-8.15)
	pRCC	4.37 (0.29-65.00)
	High pathological Stage	9.69 (2.43-38.71)
	Age>med	4.64 (0.961-22.35)
	miR-21>med	4.84 (0.641-36.52)
miR-141	Histological subtype	
	ccRCC	0.80 (0.07-9.24)
	pRCC	7.638 (0.70-82.63)
	High pathological Stage	11.25 (2.90-43.71)
	Age>med	4.07 (0.86-19.42)
	miR-141≤med	3.62 (0.62-21.05)
miR-155	Histological subtype	
	ccRCC	1.22 (0.011-13.19)
	pRCC	13.32 (1.36-130.99)
	High pathological Stage	11.90 (2.68-52.86)
	Age>med	4.83 (1.00-23.22)
	miR-155>med	1.50 (0.267-8.482)

med, median value, ccRCC, clear cell renal cell carcinoma; pRCC, papillary RCC;

Supplementary Table 4: Distribution of microRNAs expression levels among the different histological subtypes in kidney in *ex-vivo* biopsies.

	miR-141	P-Value (K-W)	miR-200b	P-Value (K-W)
Tumor subtype		**<0.001**		**<0.001**
Oncocytoma	3.0 (0.6-427.3)		49.27 (3-129.8)	
chRCC	113.5 (1.5-252.3)		49.3 (3-129.8)	
pRCC	20.2 (1.1-140.2)		333.8 (50.8-863.8)	
ccRCC	14.3 (72.4-0.1)		138.16 (4.2-573)	

ccRCC, clear cell renal cell carcinoma; pRCC, papillary RCC; chrRCC, chromophobe RCC; K-W, Kruskall-Wallis test